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Research paper

A novel method to prepare highly encapsulated interferon-α-2b containing liposomes for intramuscular sustained release

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Abstract

A novel modified film-hydration–dilution method was employed to prepare highly encapsulated interferon-α-2b containing liposomes for intramuscular sustained release. The liposomes produced by this technique were a mixture of mainly unilamellar vesicles and a small number of multilamellar vesicles. The trapping efficiency was above 80%. With at least 60-fold dilution, Triton X-100 at the concentration of 0.3% (w/v) in phosphate buffered saline (PBS) was able to solubilize phospholipids without denaturing the protein and/or interfering with the enzyme-linked immunoassay (ELISA). After three homogenization cycles under a pressure of 70 MPa the size of liposomes was reduced from 978 to 101 nm while the activity of interferon-α-2b decreased by 9.9% compared to the control. Although liposomes were physically stable for 22 months at 4 °C the mean size of the liposomes increased slightly from 101 to 122 nm. The levels of free interferon-α-2b at the site of intramuscular injection decreased rapidly with only 4.15% of initial dose retained at the injection site after 0.33 h following injection of an interferon-α-2b solution (nonencapsulated). In contrast, interferon-α-2b encapsulated in liposomes was retained at the site of intramuscular injection at higher levels than free interferon-α-2b ($p < 0.05$). Larger liposomes containing interferon-α-2b (978 nm) were the most effective for local retention because 27.8% of interferon-α-2b was retained after 24 h. These liposomes have the potential to be topically injected for treating genital herpes with prolonged interferon levels at the local injection site. Since the smaller liposomes (75.8 and 101 nm) retained interferon-α-2b at the injection site for shorter times while enhancing the blood circulation of the drug, they are potentially good carriers for systemic therapy with higher bioavailability and liver targeting.

Keywords: Liposome; Interferon-α-2b; Film-hydration–dilution; Sustained-release; Intramuscular injection

1. Introduction

Interferon-α (IFN-α) is a therapeutic protein with antiviral, antiproliferative, and immunomodulating properties [1]. Interferon enhances the tumouricidal and microbicidial activity of macrophages, which play an important role in human immune response against cancer and infections [2]. Interferon-α has been given intravenously (i.v.) or intramuscularly (i.m.). However, interferon must be frequently administered to obtain desired therapeutic effect because of its short plasma half-life [3]. From a formulation point of view, interferon is very sensitive to a variety of detrimental conditions that occur during the preparation of parenteral dosage forms, leading to a partial or complete loss of its biological activity [4,5].

Liposomes as drug delivery systems for interferon have great potential in providing controlled release, altering the bioavailability, and increasing the stability of this labile

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drug [1,6–9]. Liposomal IFN-α preparations can also alter the pharmacokinetics [10–13] and uptake of IFN-α in comparison with free IFN-α [14], result in local retention of the encapsulated material after subcutaneous injection [15–17], and increase the convenience of therapy by allowing less frequent drug administration [18]. Furthermore, encapsulation of IFN-α in liposomes may reduce the side effects while enhancing its therapeutic activity [19].

It has been reported that the composition and preparation methods of liposomes are important factors determining the levels of activity of human IFN-α and the size distribution of the liposomes [2,19]. Deborah et al. [20] have previously demonstrated that liposomal formulations of IFN-γ can be prepared with good encapsulation efficiency ($\geqslant$50%). Human IFN-γ can also be formulated in large, multi-lamellar liposomes with higher association efficiency (>80%) and preservation of bioactivity [17]. However, the trapping efficiency of interferon-α is quite low, 4–30% [1,2,6]. When homogenization was used in the preparation, the trapping efficiency for interferon-α was even lower, only around 3% [2]. After the free interferon was removed from the liposomes, the liposome-associated IFN-α (<0.2 μm) prolonged the local retention of IFN-α following intramuscular injection in mice [14]. Also, after intravenous injections in mice, the levels of IFN-α were much higher in the lungs and liver compared to those that received free IFN-α [14]. The composition of the liposomes affected the local retention and circulation of IFN-α in vivo [14].

The purpose of this study was to prepare drug-containing liposomes with higher trapping efficiency and improved stability using recombinant human interferon-α-2b (rhIFN-α-2b) as a model drug, and to investigate the effect of liposomal size on the absorption and localized retention of interferon after intramuscular administration in mice. This study was conducted also because there are no reports regarding the effect of liposome size on the local retention of rhIFN-α-2b at the injection sites or on the in vivo behavior of liposome-encapsulated interferon.

2. Materials and methods

2.1. Materials

Recombinant human interferon-α-2b (rhIFN-α-2b) from *Escherichia coli*, with a specific activity of 1.58×10^8 IU/mg of protein and with 1% of human serum albumin (HSA) as a stability agent, and recombinant human interferon-α-2b lyophilized power for injection were kindly provided by Suzhou Xinbao Pharmaceutical Group (Suzhou, PR China). Soybean lecithin for injection was purchased from Shanghai Taiwei Pharmaceutical Industry Co., Ltd (Shanghai, PR China). Cholesterol was obtained from Sigma, Dongfang Chemical Regency, China (Beijing, PR China). Polyvinylpyrrolidone K30 (PVP) was kindly donated by International Specialty Products Limited Corporation Beijing Representative Office (Beijing, PR China). Triton X-100 was purchased from Farco Chemical Supplies (Hong Kong). An ELISA kit for specific evaluation of natural or recombinant human IFN-α-2b levels was purchased from Beijing Bangding Taike Biotechnology Limited Corporation (Beijing, PR China). Other reagents were of HPLC grade and used as received.

2.2. Animals

Kunming mice (males, weighing between 22 and 25 g, obtained from the Animal Center of Shenyang Pharmaceutical University, Shenyang, PR China) were used for the in vivo absorption study. All animals were housed in cages in a temperature-, humidity-, and light-controlled room and were allowed free access to food and water throughout the study except for overnight fast prior to blood sampling.

2.3. Preparation of rhIFN-α-2b containing liposomes

rhIFN-α-2b liposomes were prepared using a novel modified film-hydration–dilution method followed by homogenization. Briefly, 2400 mg of soybean lecithin and 360 mg of cholesterol were dissolved in 50 mL of chloroform in a round bottomed flask, and dried in a rotary evaporator under reduced pressure to form a thin lipid film. The film was hydrated with 30 mL of phosphate buffered saline (PBS, pH 7.2) containing rhIFN-α-2b (4.1×10^8 IU) and PVP (5%) at 45 °C for 30 min, and then the solution was cooled down to 35 °C at room temperature. Next, the solution was hydrated with 15 mL of 2% PVP buffer solution (PBS, pH 7.2) at 35 °C for 20 min, and then further equilibrated with 38 mL of 2% PVP buffer solution (PBS, pH 7.2) for another 20 min to form liposome suspension. Finally, the liposome suspension was homogenized by a high-pressure homogenizer system (Nanomaizer, YSNM-1500, Yoshida Kikai Co., Ltd, Japan) under the automatic operation at the following conditions: air pressure 70 MPa; intake rate 100 (1–100); discharge delay time 5 (1–20); discharge rate 59 (1–100); intake delay time 4 (1–20). The homogenized valve was cooled with ice water. Three cycles are required to achieve the liposomes with the desired size distribution. The liposome dispersions were passed through a 0.22 μm membrane to eliminate any microbes and were stored at 4–8 °C.

2.4. Microscopic characterization of rhIFN-α-2b containing liposomes

rhIFN-α-2b liposomes were characterized by electron microscopy using negative staining with 1% phosphotungstic acid. The liposome sample was diluted with PBS (pH 7.2), and a drop of the diluted sample was placed on the surface of copper grid. Then the diluted sample was stained with 1% phosphotungstic acid and dried by air. The microscopic appearance of the liposomes was examined under Transmission Electron Microscopy (TEM) and micrographs were taken for each sample.

2.5. Particle-size measurements of liposomes

A laser diffraction particle analyzer (L230, Beckman Coulter, USA) was used to determine the size of liposomal rhIFN-α-2b. The sample was added until a polarization intensity differential scattering (PIDS) obscuration of 40% was obtained. The dilution medium was normal saline and the refractive index was 1.333. One hundred and twenty seconds were chosen for all measurements.

2.6. Enzyme-linked immunoassay

An enzyme-linked immunoassay (ELISA) was used to quantify the amount of rhIFN-α-2b. Briefly, 100 μL of the diluted sample of the rhIFN-α-2b liposomes or the rhIFN-α standards (ranging from 31 to 1000 pg mL^{-1}, ELISA test kit) was added to the wells (ELISA test kit) that were coated with an antibody against rhIFN-α. The wells were incubated for 1 h at 37 °C and washed six times with wash buffer (ELISA test kit). Then the conjugated secondary antibody reagent (ELISA test kit) was added to each well, and the wells were incubated at 37 °C for 45 min. Next, the wells were washed six times with wash buffer, and incubated with 100 μL of substrate solution (ELISA test kit) at 37 °C for 15 min. Finally, the reaction was stopped by adding 50 μL of stop solution (ELISA test kit), and the optical density (OD) was determined at 490 nm within 30 min. Both standards and samples were assayed in duplicate, and a separate standard curve was run in each assay. The linear range for ELISA was 31–1000 pg mL^{-1}, the average inter-plate and intra-plate variability was less than 10%, and the sensitivity was 31 pg mL^{-1}.

2.7. Liposome solubilization by Triton X-100 and its effect on the activity of rhIFN-α-2b

Liposomes have to be disrupted to release the associated drug before ELISA determination because the encapsulated rhIFN-α-2b cannot be detected. In this study, blank liposomes were diluted with water (control) or 0.3% (w/v) Triton X-100 buffer solution (PBS, pH 7.2) and incubated for 30 min at room temperature. The disappearance of turbidity (absorbance ≈ 0) of liposome dispersion was considered as an indication of vesicle solubilization. The absorbance of liposome dispersions was measured at 480 nm using a Shimadzu UV-1700 Spectrophotometer (Shimadzu Corp., Kyoto, Japan). The results showed that using Triton X-100 buffer solution (PBS, pH 7.2) at the concentration of 0.3% (w/v), with at least 60-time dilution, the liposomes were dissolved and the drug molecules were released from the vesicles (Table 1).

To test whether Triton X-100 affects the ELISA determination [21], rhIFN-α-2b was diluted with 0.3% (w/v) Triton X-100 buffer solution (PBS, pH 7.2) or dilution buffer (ELISA test kit) to the same concentration (approximately 500 pg mL^{-1}), and was incubated for 30 min. The activity of rhIFN-α-2b was determined using a standardized ELISA method. Our ELISA showed there was no significant difference in rhIFN-α-2b content regardless of using buffer (ELISA test kit, 521 pg mL^{-1}, $n = 3$) or 0.3% (w/v) Triton X-100 buffered solution (PBS, pH 7.2, 533 pg mL^{-1} $n = 3$). Therefore, 0.3% (w/v) Triton X-100 did not affect the activity of rhIFN-α-2b.

Table 1
Absorbance of liposomes after dilution

Dilution medium	Dilution factor			
	10	20	60	1000
Water	0.800	0.668	0.206	0.019
Triton X-100[a]	0.281	0.024	0.001	−0.001

[a] 0.3% (w/v) buffer solution (PBS, pH 7.2).

2.8. Antiviral activity of rhIFN-α-2b liposomes

The antiviral activity of rhIFN-α-2b was measured with a bioassay according to China Biologicals Requirements, utilizing the property of interferon to protect certain cell lines from the destruction by virus. rhIFN-α-2b was titrated by 50% cytopathic effect reduction method using vesicular stomatitis virus and human amnionic cells (WISH). The effect was determined by measuring the cellular uptake of neutral red dyes using auto-reader at 570 nm. All the assays were made employing the international reference preparations for human interferon-α obtained from the National Institute for Biological Standards and Control (Beijing, PR China). All titres were reported in IU mL^{-1}.

2.9. The trapping efficiency of rhIFN-α-2b in liposomes

Free and liposomal rhIFN-α-2b (entrapped and adsorbed) were separated by ultracentrifugation which was performed for 2 h at 126,000*g* and a temperature of 10 °C. The supernatant was collected, diluted with 0.3% (w/v) Triton X-100 buffer solution (PBS, pH 7.2) to a drug concentration of approximately 500 pg mL^{-1}, and then assayed using ELISA to determine the amount of free drug. The total amount of rhIFN-α-2b in liposomes was determined by the following procedure: the liposomal rhIFN-α-2b was dissolved and diluted with 0.3% Triton X-100 buffer solution (PBS, pH 7.2) to about 500 pg mL^{-1}, incubated at 10 °C for 30 min, and then assayed by ELISA. The trapping efficiency was calculated by the following equation:

$$\text{Trapping efficiency (\%)} = \frac{100 \times (\text{total amount of drug} - \text{amount of free drug})}{\text{total amount of drug}}. \quad (1)$$

2.10. Levels of rhIFN-α-2b in thighs

Animals were randomly assigned into four groups to provide local retention evaluation. Group 1 (30 mice) was injected intramuscularly 0.05 mL of rhIFN-α-2b reconstituted

solution (37.4 μg mL^{-1}) in right thighs. Groups 2–4 (50 mice in each group) received intramuscularly 0.05 mL of liposomal rhIFN-α-2b (37.4 μg mL^{-1}) in right thighs with different liposome sizes of 75.8, 101, and 978 nm, respectively. Five mice from each group were sacrificed at certain time intervals. The thigh muscles were excised, quick frozen on dry ice, and stored in freezer (−80 °C) until assayed.

The levels of rhIFN α-2b in the thigh muscles were determined by ELISA as described above. The tissue samples were homogenized in 0.3% (w/v) Triton X-100 buffer solution (PBS, pH 7.2) as 10% (w/v) suspensions, and then centrifuged at 10,000 rpm for 20 min. The supernatant was assayed for rhIFN-α-2b levels using ELISA.

2.11. Statistical analysis

The remaining rhIFN-α-2b at the injection site with 5 replicates was evaluated using a one-way ANOVA (SAS Institute, Inc., Cary, NC, USA). Post hoc comparisons of the means of individual groups were performed using DUNCAN's test. A p value of <0.05 denoted significance in all cases.

3. Results and discussion

3.1. Characterization of rhIFN-α-2b containing liposomes prepared by homogenization

One major barrier to the increased application of liposomal drug delivery systems lies in the low trapping efficiency of drug and low stability of liposomes in most cases. In this study, we were able to prepare reproducible liposomal rhIFN-α-2b with trapping efficiency higher than 80% (Table 2). The size distribution of rhIFN-α-2b liposomes prepared after three homogenization cycles had a mean size of 101 nm with a narrow size distribution (Table 3). TEM micrographs (Fig. 1) showed that the liposomes obtained after three homogenization cycles were mixture of mostly single layer vesicles and a few multilayer vesicles.

3.2. Effect of homogenization frequency on particle size distribution and activity of liposomal rhIFN-α-2b

Both the size of the liposomes and the activity of interferon-α-2b were affected by the number of homogenization cycles (Table 3). The size of liposomes was reduced while the activity of interferon-α-2b was decreased as the number of homogenization cycles increased. The size distribution of the liposomes had a mean size of 101 nm after three homogenization cycles under a pressure of 70 MPa while the activity of interferon-α-2b was decreased by 9.9% compared to the control. Increasing the number of homogenization cycles to four decreased the mean size of the liposomes further to 75.8 nm with a further loss in activity equal to 18.6%. Based on these results it seems that 100 nm was an optimum size for the liposomes ensuring limited loss in activity. In addition, the liposomes containing rhIFN-α-2b were physically stable for 22 months at 4 °C, the mean size increasing slightly from 101 to 122 nm (Table 3).

Table 2
The size distribution and trapping efficiency of liposomal rhIFN-α-2b

Batch	Distribution peak (SD) (nm)	d_{90}[a] (nm)	% Trapping efficiency (SD)[b]
1	98 (18)	123	81.1 (1.8)
2	96 (17)	119	84.6 (1.1)
3	102 (19)	128	82.9 (1.3)

[a] The value is the particle size in diameter that corresponds to 90% of all particles in cumulative volume distributions.
[b] $n = 3$.

Table 3
Effect of the number of homogenization cycles on particle size and activity of liposomal rhIFN-α-2b

The number of homogenization cycles	Distribution peak (SD) (nm)	d_{90} (nm)	Activity (×10^6 IU mL^{-1})
0	978 (454)	–	4.84
1	216 (28)	254	4.78
2	143 (28)	176	4.50
3	101 (17)	123	4.36
	122[a] (22)	152[a]	
4	75.8 (17)	99.5	3.94

[a] *Note:* Data measured after storage at 4 °C for 22 months.

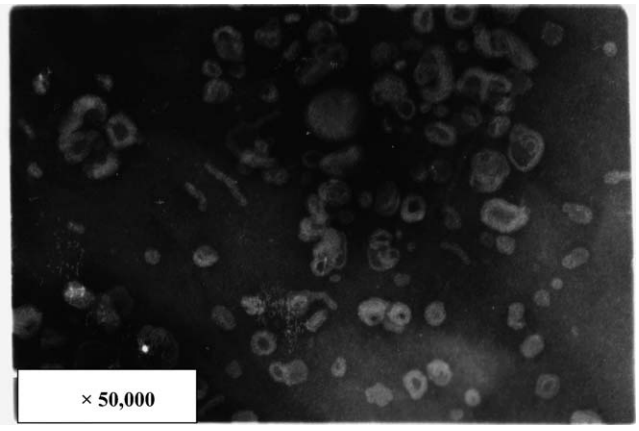

Fig. 1. TEM micrographs of rhIFN-α-2b containing liposomes.

3.3. Trapping efficiency of rhIFN-α-2b liposomes

To make liposomes containing polypeptides or large molecules with high trapping efficiency, the critical factor is the preparation process. In this study, a novel modified film-hydration–dilution method was used to prepare the liposomes. This method was based on thin film preparation [2,22,23] and employed a multi-step hydration–dilution technique to make a liposome suspension. Unlike the one-step hydration, i.e., adding all the required hydration medium containing the drug into the film to form liposomes, the hydration procedure used here was divided into

three steps: (1) mixing hydration medium (PBS) containing rhIFN-α-2b and PVP (5%) with the film to make a viscous liposome gel in which the drug was encapsulated into liposomes; (2) adding a hydration medium (2% PVP in PBS) to the gel at a temperature below the lipid phase transition temperature; (3) further hydration by adding more hydration medium (2% PVP in PBS) to the now hydrated gel again at a temperature below lipid phase transition temperature. PVP was added to the hydration medium because it improved the trapping efficiency and increased the physical stability of liposomes.

Using this film-hydration–dilution method, liposomes containing interferon-α-2b with encapsulation efficiencies higher than 80% were prepared reproducibly (Table 2). This technique was also used to prepare liposomes of other drug molecules including interleukin-2, recombinant human interferon-γ, doxorubicin, and berberin hydrochloride. Stable liposomes were prepared with these drugs. The highest encapsulation efficiency was 93% for doxorubicin, and lowest 78% for berberin hydrochloride. These results indicated that this novel modified film-hydration–dilution method has wide applicability.

3.4. Local retention of rhIFN-α-2b liposomes in vivo

The retention of liposome-associated rhIFN-α-2b at the sites of intramuscular injection in mice was compared with free rhIFN-α-2b. As shown in Fig. 2, the levels of free rhIFN-α-2b at the site of injection decreased rapidly within 4 h following injection. There was only 4.15% of initial dose was retained at the injection site 0.33 h following injection of rhIFN-α-2b solution, and no interferon-α-2b activity was detected in the muscles after 8 h, which agreed well with previous reports [24]. In contrast, rhIFN-α-2b encapsulated in liposomes was retained at the site of injection at initially higher levels than free interferon ($p < 0.05$) (Fig. 2), which agreed with previous reports [1,15,16].

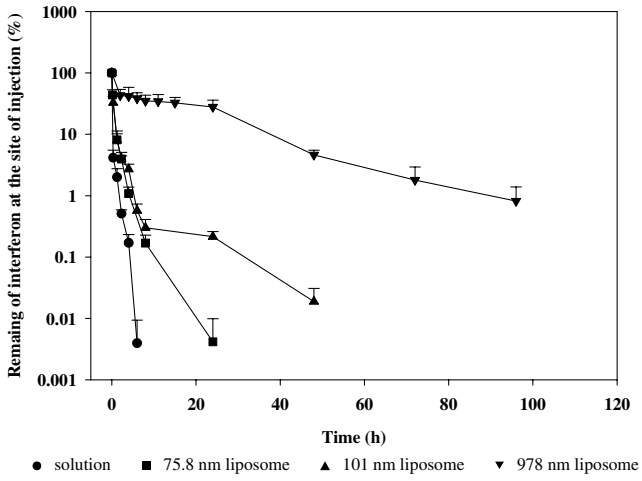

Fig. 2. Reduction in the level of interferon at the injection site after administration of rhIFN-α-2b solution and liposomes with decreasing particle sizes.

3.5. Effect of the liposomal size on the local retention of rhIFN-α-2b

It has been reported that the particle size of liposome is a critical factor in determining its residence time at the injection site [25]. Several reports refer to a cutoff value of 100 nm, above which the liposomes do not appear in the blood to any substantial extent because it remains at the injection site [26–29]. This study also showed that larger liposomes (978 nm) were retained at the injection site longer (27.8% of rhIFN-α-2b retained after 24 h). The drug was also slowly absorbed from the injection site because the local level of the rhIFN-α-2b was still 4.61% 48 h after intramuscular injection. This was comparable to the peak levels of 4.15% obtained 0.33 h after free interferon-α-2b was injected. The local retention of rhIFN-α-2b was less following the administration of smaller liposomes (101 nm) because local rhIFN-α-2b level was 3.96% after 2.3 h post injection. This was similar to the peak levels of 4.61% obtained 48 h after injecting the larger liposomes (978 nm). Reducing the liposome size from 101 to 75.8 nm did not decrease local retention times ($p > 0.05$). These results showed that the smaller liposomes ($\leqslant$100 nm) resulted in intermediate therapeutic levels of rhIFN-α-2b at the injection sites.

3.6. Possible cause of local retention

The differences in local retention might be due to differences in the absorption rate and route for liposomes with different size distributions [25]. Another possibility is that free interferon may be less stable than interferon trapped in vesicles at the injection site. However, preliminary studies showed that there was no significant difference between the stability of free rhIFN-α-2b and liposomal rhIFN-α-2b at the injection site.

Unlike the free interferon, which was absorbed into the blood circulation mainly via the lymphatic capillaries after injection, interferon liposomes would stay at the injection site or be absorbed through the lymph systems around the injection site. Smaller vesicles ($\leqslant$100 nm) containing rhIFN-α-2b may be taken up by the lymph capillaries draining the injection sites and passed through the lymph nodes to reach the general circulation [25], where they behave as if administered by the i.v. route. Once liposomes have reached the blood compartment, they tend to accumulate in organs rich in cells of the mononuclear phagocyte system (MPS) such as the liver and spleen [29]. The accumulation of rhIFN-α-2b in the liver is an advantage for the treatment of hepatitis B since IFN-α is the only effective drug for it [19]. Larger liposomes remain at the injection site until the liposomes are degraded. Then the drug is released and absorbed. With larger liposomes drug concentrations in the blood compartment are usually low since drug release and subsequent absorption from the injection site is slow. Particularly drugs that are rapidly cleared, such as proteins and peptides, do not accumulate in the blood compartment, because clearance

from the blood is faster than absorption from the injection site.

3.7. A balance between the vesicle size and the local retention time of liposomal rhIFN-α-2b

Although the larger the particle the greater the retention was, there must be a balance between the particle size and the retention time. Larger liposomes degrade slowly at the injection sites; release the free drug molecules into blood circulation where they are easily destroyed by the enzymes in the circulation system. The elimination half-life of drug cannot be prolonged. On the other hand, for liposomes $\leqslant$100 nm, although their retention time at injection site is shorter than large ones, the drug molecules stay encapsulated. These vesicles will take the drugs through the lymph systems into the blood circulation. Due to protection in these vesicles, drug molecules would not interact directly with the enzymes in vivo. Thus, the in vivo stability is increased, resulting in a longer half-life, higher bioavailability and higher levels in the liver. Therefore, the size distribution of liposomes is the key factor for topical injection of interferon liposomes.

Larger liposomes (>100 nm) can only act as sustained reservoir at the topical injection site, while liposomes ($\leqslant$100 nm) not only provide a certain sustained absorption, but also stabilize the drug during in vivo transport and in blood circulation and are targeted to mononuclear phagocyte system (MPS) such as the liver and spleen. Therefore, larger liposomes may be topically injected for the therapy of genital herpes, since they could keep a higher topical concentration of rhIFN-α-2b. On the other hand, smaller liposomes ($\leqslant$100 nm) may be good carriers for systemic therapy, especially for virus hepatitis, because they could lead to higher bioavailability and liver targeting.

4. Conclusions

This study showed that rhIFN-α-2b liposomes with high trapping efficiency can be prepared using a novel modified film-hydration–dilution method employing PVP which played an important role in improving the encapsulation of the drug and stability of liposomes. In this preparation process homogenization stress affects the size of the liposomes and the activity of interferon-α-2b. The size of the liposome is important because it influences the local retention of interferon-α-2b. The larger the size, the greater the retention was. However, smaller liposomes ($\leqslant$100 nm) prepared by this technique still provided intermediate retention at the injection site with the added benefits of decreased loss of the interferon-α-2b activity and improved liver targeting.

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