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ORIGINAL RESEARCH

Preparation of bioactive interferon alpha–loaded polysaccharide nanoparticles using a new approach of temperature-induced water phase/water-phase emulsion

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Abstract: The aim of this study was to develop a temperature-induced polyethylene glycol (PEG) water phase/polysaccharide water-phase emulsion approach for preparing interferon alpha-2b (IFNα-2b)-loaded polysaccharide nanoparticles. IFNα-2b was first added to a mixture of an aqueous solution of PEG and polysaccharide. The mixture solution was stirred in a magnetic stirrer at a rate of 2000 rpm for 45 seconds at 0°C ± 0.5°C. The solution was then prefrozen at different temperatures. The polysaccharide and IFNα-2b partitioned in the polysaccharide phase were preferentially separated out as the dispersed phase from the mixture solution during the prefreezing process. Then the prefrozen sample was freeze-dried to powder form. In order to remove the PEG, the powder was washed with dichloromethane. Once IFNα-2b was loaded into the polysaccharide nanoparticles, these nanoparticles could gain resistance to vapor–water and water–oil interfaces to protect IFNα-2b. The antiviral activity of the polysaccharide nanoparticles in vitro was highly preserved (above 97%), while the antiviral activity of IFNα-2b–loaded polysaccharide nanoparticles using the control water-in-oil-in-water method was only 71%. The antiviral activity of the IFNα-2b from blood samples was also determined on the basis of the activity to inhibit the cytopathic effects of the Sindbis virus on Follicular Lymphoma cells (FL). The antiviral activity in vivo was also highly preserved (above 97%). These polysaccharide nanoparticles could be processed to different formulations according to clinical requirements.

Keywords: activity of interferon alpha-2b, interferon alpha-2b, stability of interferon alpha-2b, dextran, nanoparticles

Introduction

In clinical studies, interferon alpha-2b (IFNα-2b) is widely used in hepatitis B, hepatitis C, leukemia, multiple myeloma, and carcinoma therapy.[1–4] As its half-life in serum is short and its therapeutic index is relatively narrow, the drug is frequently injected clinically. This manner of administration often results in fluctuating blood drug concentration, which leads to substantial and inevitable toxicity. Thus, targeted delivery (eg, liver) or controlled delivery is necessary for IFNα-2b in order to avoid side effects and achieve therapeutic effects. A polyethylene glycol (PEG)ylated long-efficacy IFN has been developed, but PEGylated IFNα-2b induces side effects more easily than native IFNα-2b. A sustained or controlled drug-delivery system has also been used for IFNα-2b delivery,[3–10] but it often results in activity lost and incomplete release[11–17] because of these formulations not avoiding water–oil interfaces and other factors.

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Spray-drying, antisolvent precipitation, spray-freeze–drying, and supercritical fluid technology have been investigated for preparing protein nanoparticles.[18–20] However, due to pressure, interfaces, heating, and organic solvents involved in different manufacturing steps, these methods are potentially detrimental to the structures and functions of proteins.[21] Other methods, such as manufacturing approaches (emulsion methods) for polymer nanoparticles (including nanogels), inorganic nanoparticles, and liposomes have been developed for preparing protein nanoparticles. However, some also expose the hydrophobic cores, leading to protein instability (eg, poly(lactic-*co*-glycolic acid) [PLGA], PLGA–PEG nanoparticles), burst release, irregular nanospherical shapes or low encapsulating efficiency (eg, nanogel, liposomes, polymersomes), and incomplete release.[22–33] We have studied a solution that avoids vapor–water and water–oil interfaces for the preparation of bioactive protein microparticles,[19,34–37] but the solution cannot be prepared for nanoscale particles, which has resulted in no further preparation of a targeted-delivery protein system.

In this study, we developed a temperature-induced PEG water-phase/dextran water-phase emulsion method for the manufacture of nanoscale-sized, IFNα-2b–loaded dextran particles. The IFNα-2b–loaded nanoparticles were evaluated both in vivo and in vitro. A high activity and high encapsulation efficiency loading of IFNα-2b into nanoparticles achieved by a low temperature–induced PEG water-phase/dextran water-phase emulsion method by the assembly of IFNα-2b–partitioned dextran nanoparticles.

Materials and methods

Materials

IFNα-2b was obtained from Schering-Plough (Kenilworth, NJ). A human IFNα-2b enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN). The polysaccharide (dextran, molecular weight [MW] 64,000–76,000 Da, biochemical reagent) and polyethylene glycol (PEG, MW 6000 Da, biochemical reagent) were purchased from Sigma-Aldrich (St Louis, MO). Fetal bovine serum was purchased from HyClone Lab (Logan, UT). Minimum essential medium was obtained from MediaTech (Herndon, VA).

Animals

Eight-week-old female Sprague Dawley rats weighing approximately 60 ± 10 g were used. The mice were raised under standard conditions at 22°C ± 2°C. The animal experiments complied with laboratory animal care principles and were subject to approval by the Institutional Animal Care and Utilization Committee of Shanghai Jiao Tong University.

Preparations of IFNα-2b–loaded dextran nanoparticles

The mixture solution (3.0 mL) containing 0.1% (w/w) IFNα-2b, 0.5% (w/w) dextran (MW 64,000–76,000 Da), and 4.5% PEG (MW 6000 Da) was stirred on a magnetic stirrer at a rate of 2000 rpm for 45 seconds in a 5-mL vial, and the vial was placed in a glass beaker containing 100 g ice and 200 mL water, and then prefrozen in three different refrigerators of –10°C, –20°C, and –80°C for 12 hours. The prefrozen samples were freeze-dried using a Christ (Osterode, Germany) Alpha 1–2 laboratory freeze-dryer operating at 5.20×10^{-3} Pa for 24 hours. The freeze-dried powder was suspended and washed in 5.5 mL dichloromethane (DCM), and then the suspension was centrifuged at 12,000 ± 200 rpm for 5 ± 0.5 minutes in an Anke (Shanghai, China) TGL-16C centrifuge to remove the continuous phase of PEG. The pellet was used for further processing. The suspending-washing-centrifugation step was repeated four times, the particles obtained were dried to remove solvent residues under 1.33 ± 0.03 Pa for 24 hours using a vacuum dryer (DZF-3; Fuma, Shanghai, China). Less than 0.5% (w/w) of the PEG residual in the collected particles was subjected to the process.[38] For the control method (water-in-oil-in-water, [W/O/W]), a solution with 0.1% (w/w) IFNα-2b and 0.8% (w/w) dextran (MW 64,000–76,000 Da) was placed into a 6-mL vial and stirred in the magnetic stirrer at a rate of 2000 rpm for 45 seconds, and then the solution was dispersed into 4.5 mL 7.2% (w/w) PEG DCM solution, with 1 minutes' mixing. Then, the mixture was lyophilized using liquid nitrogen and freeze-dried, and the dextran nanoparticles were obtained after having removed the PEG.

Scanning electron microscopy images

Scanning electron microscopy (SEM) of protein-loaded polysaccharide nanoparticles was performed using a Sirion 200 SEM (FEI, Hillsboro, OR). All the nanoparticles were placed on double-sided tape, which was attached to a stub of metal and sprayed using a gold vapor for 12 minutes under an argon atmosphere. The SEM images were taken at a sputtering energy of 5 kV under high vacuum.

Size distribution and zeta potential of dextran nanoparticles

The size distribution and average naoparticle size of dextran nanoparticles were performed using a Particle Size Analyzer

and Particle Shape Analyzer (CIS-100; Ankersmid, Edegem, Belgium). Ten mg dry dextran nanoparticles were dispersed in the quartz cell containing 0.5 mL isopropanol. Zeta potential of the nanoparticles was measured using a Zetasizer Nano ZS (M3-PALS; Malvern Instruments, Malvern, UK).

Determination of the IFN-2b aggregations from nanoparticles

Size-exclusion chromatography (SEC) was carried out with a TSK G2000SWXL size-exclusion column (Shimadzu, Tokyo, Japan) and a high-performance liquid chromatography (HPLC) system. The mobile phase was analyzed using a peristaltic pump with a phosphate-buffered saline solution of 50 mM (pH 7.4) and an 0.8 mL/minute flow rate at 25°C ± 2.5°C. The absorbance of each sample was determined at a wavelength of 214 nm, because the dextran and PEG have no absorbance at this wavelength; only IFNα-2b does, and IFNα-2b absorbance at 214-nm wavelength has higher sensitivity than at 280 nm. The retention times for monomer peak and aggregation absorbance peak of IFNα-2b were found to be 15.0 minutes and 13.5 minutes, respectively. The amount of IFNα-2b, whether aggregated or still a monomer, was calculated based on the peak area of IFNα-2b monomer absorbance peak areas at a wavelength of 214 nm (retention at 15.0 minutes), divided by the total peak area, which was that monomer absorbance peak area plus the aggregation absorbance peak area (retention at 13.5 minutes).

Determination of the IFNα-2b content and antiviral activity from nanoparticles

Six-mg dextran nanoparticle preparations encapsulating IFNα-2b were weighed out for six vials. Then, the nanoparticles of each vial were dissolved in 1 mL minimum essential medium supplemented with 5% fetal bovine serum and repeated three times. Protein content (IFNα-2b) in the respective nanoparticle preparations was calibrated by the specific ELISA for the three repeated times. The antiviral activity of the IFN-α was determined on the basis of the activity of inhibiting the cytopathic effects of the Sindbis virus on follicular lymphoma cells (FL).[39]

Determination of IFNα-2b encapsulation efficiency

Five-mg nanoparticles were suspended in 10 mL DCM and then stirred in the magnetic stirrer at a rate of 2000 rpm for 5 minutes. The above suspension was centrifuged at 12,000 ± 200 rpm for 5 ± 0.5 minutes on an Anker TGL-16C centrifuge to remove the unencapsulated IFNα. The nanoparticles' IFNα-2b encapsulation efficiency was calculated as described in the "Determination of the IFNα-2b content" section.

Storage stability of the IFNα-2b from dextran nanoparticles

Temperature was often a factor that affected IFNα-2b stability. Because protein storage temperature, room temperature, and in vivo normal temperature were often considered to be 4°C, 25°C, and 37°C, respectively, we tested the stability of IFNα-2b from nanoparticles stored at these above temperatures. IFNα-2b-loaded nanoparticles (5 mg) were placed in a sealed vial (5 mL). Some vials were placed in 4°C for 3, 6, 9, 12, 18, 24, and 36 months; some vials were in 25°C for 3, 6, 9, 12, 18, 24, and 36 months; and the other vials were in 37°C for 3, 6, 9, 12, 18, 24, and 36 months. The reconstituted protein aggregating content from nanoparticles was measured as described in the "Determination of the IFNα-2b content and antiviral activity from nanoparticles" section.

In vivo study

The pharmacokinetic properties of IFNα from the respective dextran nanoparticle preparations were investigated in Sprague Dawley rats (4-week-old females, Shanghai Laboratory Animal Center, Shanghai, China). 5 mg nanoparticles each one suspended in 100 μL citrate buffer solution was administered by subcutaneous injection and 100 μL physiological saline and free IFNα-2b nanoparticles was also administered by subcutaneous injection as control of untreated IFNα-2b. Blood samples were taken from the tail vein at 1, 3, 6, and 24 hours postadministration, and the sum of IFNα-2b antiviral activity in 1-, 3-, 6-, and 24-hour postadministration blood samples was considered as total antiviral activity. The antiviral activity of the IFNα from blood samples was determined on the basis of the activity to inhibit the cytopathic effects of the Sindbis virus on FL cells.[39]

Statistical analysis

Data were given as means ± standard error of mean for statistical comparison; *t*-test or one-way analysis of variance using Tukey's test was employed. Statistical significance was $P < 0.05$.

Results and discussion

Morphology and size distribution

Figure 1 shows the SEM of IFNα-2b–loaded dextran nanoparticles. The particles possessed smooth surfaces,

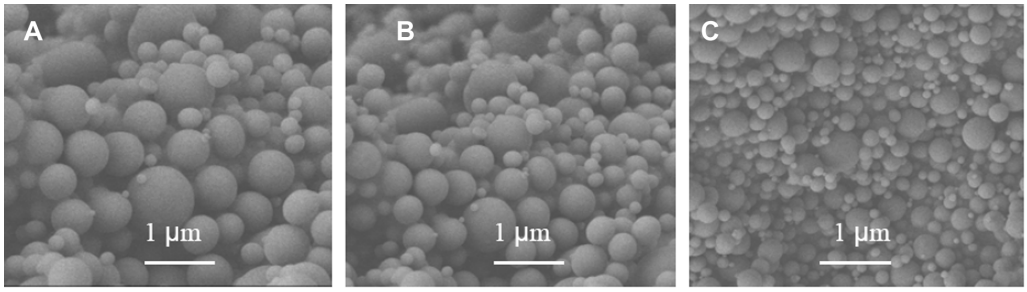

Figure 1 A–C Scanning electron microscopy of IFNα-2b–loaded dextran nanoparticles. (**A**) –10°C; (**B**) –20°C; (**C**) –80°C.

a spherical shape, and diameters ranging from 1 μm to hundreds of nm. The size of the nanoparticles decreased and their size distribution became narrower in the sequence of –10°C, –20°C, and –80°C (Table 1), because the size of the self-assembled dextran nanoparticles was limited by the decrease in temperature. We were able to control the size of the dextran particles and prepare the nanoscale IFNα-2b–loaded dextran particles using the low temperature–induced PEG water-phase/dextran water-phase emulsion method. This was because the temperature might have adjusted so that the dispersed phases (dextran phases) were generated during the freezing-induced phase-separation process (namely, formed water phase emulsion process) and the water-phase emulsion decided the dextran particle size. If the PEG water-phase and dextran water-phase separation occurred at a relatively higher temperature (th– °C system), the dextran dispersed phases would hav had m re chance to fuse with each other and form larger parti s prior to reaching a frozen state.

IFNα-2b–encapsulation efficiency

IFNα-2b–encapsulation efficiency was detected with the IFNα-2b ELISA kit. We found that encapsulation efficiency was above 98% and loading capacity 16.2% ± 1.5%, except for controlled dextran nanoparticles (only 70%) using the W/O/W method (Figure 2; the samples of A, B, and C were the same as in Figure 1A–C; D: control method [W/O/W]). This was because IFNα-2b is a water-soluble protein and easy to load into the dextran nanodroplets by preferential partition favoring the dextran phase; this was similar to the so-called aqueous two-phase system comprising a dextran and a PEG block phase.[38] The controlled method produced a lot of vapor–water and oil–water interfaces, and these interfaces resulted in IFNα-2b aggregation, absorption on PEG, and dissolving DCM. We detected the IFNα-2b of the DCM solution and confirmed that the content of the controlled method was more than 20% of the presented method. The PEG content in the samples was found to be below 5%. The protein solutions were in the microencapsulation process; the protein macromolecules might have been exposed again to oil–water interfaces, and this could have caused both aggregation and reduced loading efficiency of the proteins.[37] When IFNα-2b water solution were encapsulated into dextran nanoparticles by W/O/W, IFNα-2b directly exposed to water–oil interfaces, in addition to IFNα-2b water solution producing high osmotic pressure, resulted in protein aggregate and diffusing to continue phase.

Table 1 Average particle size and zeta potential of nanoparticles prepared in different temperatures using temperature-induced water phase/water-phase emulsion (n = 3)

Temperature	Particle size (nm)	Polydispersity index	Zeta potential
–10°C	702 ± 163	0.732 ± 0.121	–5.6 ± 2.1 mV
–20°C	401 ± 104	0.652 ± 0.134	–6.3 ± 2.2 mV
–80°C	200 ± 51	0.542 ± 0.102	–7.6 ± 3.3 mV

IFNα-2b aggregation study

Protein integrity during the preparation processes may be used to evaluate the form of protein aggregates.[40] Therefore, in order to determine any change in the IFNα-2b subjected to the different procedures, we evaluated these samples using SEC-HPLC. A 1-mg sample was dispersed in 0.5 mL citrate

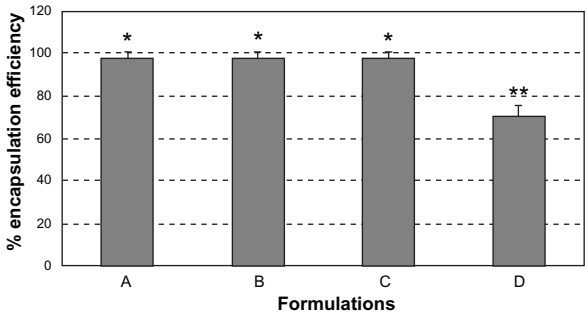

Figure 2 Encapsulation efficiency from IFNα-2b–loaded dextran nanoparticles (n = 5).
Notes: *P >0.05; **P < 0.05.

buffer solution. A 0.1-mL sample was injected into the SEC-HPLC system, as per the "Determination of the IFNα-2b aggregations from nanoparticles" section. Samples A, B, C, and D were the same as in Figures 1A–C and 2D. The IFNα-2b from the dextran nanoparticles was redissolved in an appropriate volume of phosphate buffer and assayed using SEC-HPLC. The monomers of IFNα-2b from these samples using the presented method were equaled to the monomers of nature standard IFNα-2b. The monomers of IFNα-2b from controlled samples were below 70% using the W/O/W method (Figure 3). This was because the controlled method produced a lot of water–oil or air–water interfaces, and the interfaces often caused IFNα-2b aggregation, leading to lost activity and adsorption on the materials. The glassy dextran particles and low temperature can also protect the stability and activity of protein.[37]

Antiviral activity of IFNα-2b from nanoparticles

To determine the antiviral activity of the different samples, IFNα-2b was recovered from the formulations according to the solution used for IFNα-2b encapsulation efficiency determination. The in vitro antiviral activity of the encapsulated IFNα-2b was determined on the basis of the activity to inhibit the cytopathic effects of the Sindbis virus on FL cells.[39] Samples A, B, C, and D were the same as in Figures 1A–C and 2D. The antiviral activity of the nanoparticles using the reported method was 20% more than that of the controlled method (W/O/W) (Figure 4). IFNα-2b easily aggregated and lost antiviral activity during the preparation process, which involved deleterious factors such as intense shear force, organic solvents, and high temperature. This was because IFNα-2b lost activity more easily in the oil–water interface than the water phase/water phase.[37] The controlled method (W/O/W) produced a larger amount of

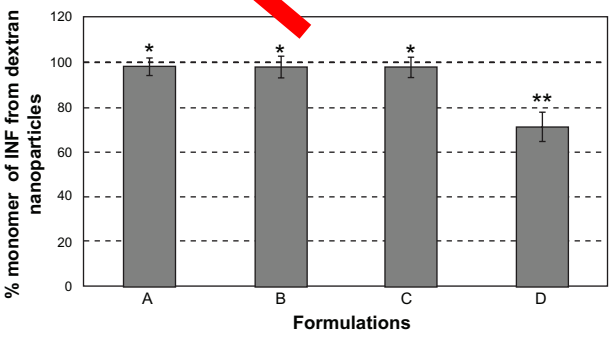

Figure 3 Percentage of monomers recovered from IFNα-2b–loaded dextran nanoparticles (n = 5).
Notes: *$P > 0.05$; **$P < 0.05$.

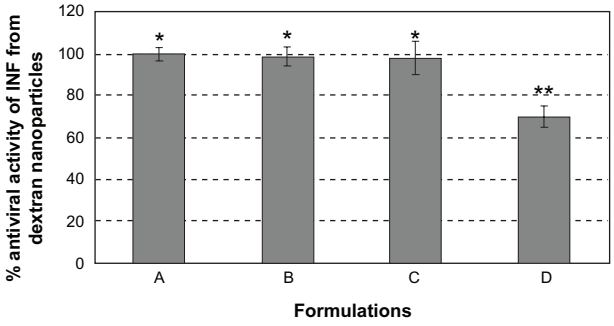

Figure 4 Antiviral activity from IFNα-2b–loaded dextran nanoparticles (n = 5).
Note: *$P > 0.05$.

oil–water interfaces at which IFNα-2b easily aggregated and lost antiviral activity. For several years dextran was the safest plasma substitute for patients who bled alot, due to it's molecular weight, and clinical trials confirmed that it was almost immunogenicity.

Stability of IFNα-2b from nanoparticles

IFNα-2b easily lost antiviral activity and aggregates during polymer nanoparticle based on the manufacturing process, which included deleterious conditions such as interfaces of organic solvents and water (or air and water), intense shear force, and high temperature.[37] The IFNα-2b stability in the size of 200 ± 51 nm nanoparticles (the sample from Figure 1C) was evaluated using antiviral activity. The antiviral activity of the IFN-α was determined on the basis of the activity to inhibit the cytopathic effects of the Sindbis virus on FL cells. The activity was obtained through blank correction (untreated IFN-α group). We found that IFNα-2b could preserve high antiviral activities at different storage temperatures (4°C, 20°C, and 37°C) (Figures 5–7). This was because when IFNα-2b was loaded with dextran nanoparticles, the nanoparticles were able to preserve the

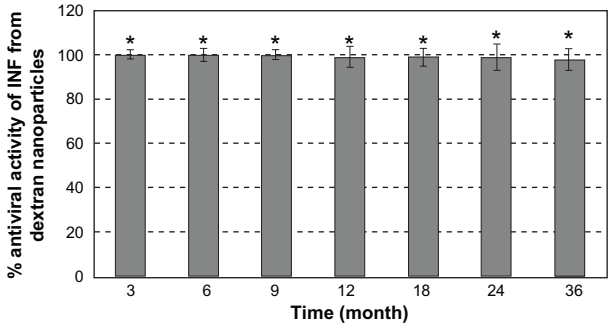

Figure 5 Stability of IFNα-2b–loaded dextran nanoparticles at 4°C (n = 5).
Note: *$P > 0.05$.

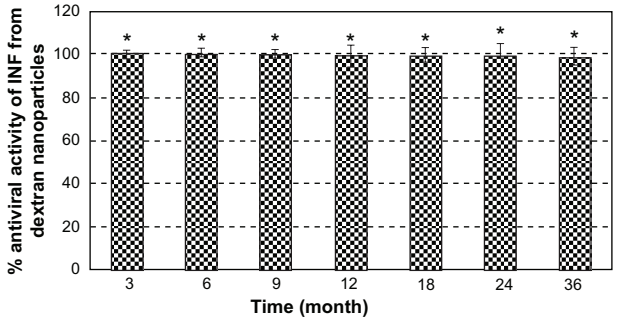

Figure 6 Stability of IFNα-2b–loaded dextran nanoparticles at 20°C (n = 5).
Note: *$P > 0.05$.

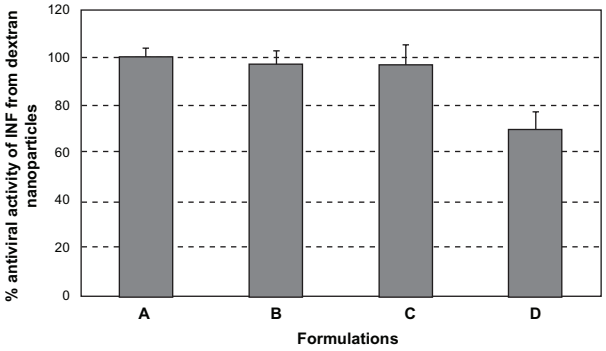

Figure 8 Experiments to determine the antiviral activity in mouse plasma following the administration of IFNα-2b–loaded dextran nanoparticles (in vivo).

IFNα-2 protein activity against the effect of temperature. Lyophilized IFNα-2b can be stable at room temperature for 3 weeks, but it should be stored in desiccate form below –18°C. IFNα-2b is often unstable when it is stored at room temperature. In order to evaluate the long-term stability of IFNα-2b, the dextran nanoparticle–loaded IFNα-2b was stored at different temperatures (4°C, 20°C, and 37°C) and for different periods (3, 6, 9, 12, 18, 24, and 36 months), and the antiviral activities of the recovered IFNα-2b from the samples was determined. The results showed that the antiviral activities of IFNα-2b were almost lost (Figures 5–7). The protein-protective mechanisms occurred because the dextran nanoparticles could provide a glassy state, and the glassy matrix could decrease the encapsulated IFNα-2b mobility and was more resistant to stresses of temperature and moisture.[19,37,41,42]

In vivo efficacy study

As shown in Figure 8, the antiviral activity of INFα-2b from nanoparticles through low temperature induced water phase/water-phase emulsion was nearly equal to that of native INFα-2b solution, whereas INFα-2b from the W/O/W method showed only 70% antiviral activity in mouse plasma after the administration of INFα-2b loaded dextran nanoparticles. These results confirmed that the dextran nanoparticles could also preserve protein activity in vivo.[19,37] This was because the controlled methods (W/O/W) produced a larger amount of oil–water interfaces at which IFNα-2b easily aggregated and lost antiviral activity.[19,37,41,42]

Samples A, B, C, and D were the same as in Figures 1A–C and 2D. The antiviral activity of the IFN-α was determined on the basis of the activity to inhibit the cytopathic effects of the vesicular virus on FL cells. The activity was obtained through blank correction (untreated IFN-α group). This was because the glassy dextran nanoparticles formed effectively protect proteins against temperatures and organic solvents.[44–48]

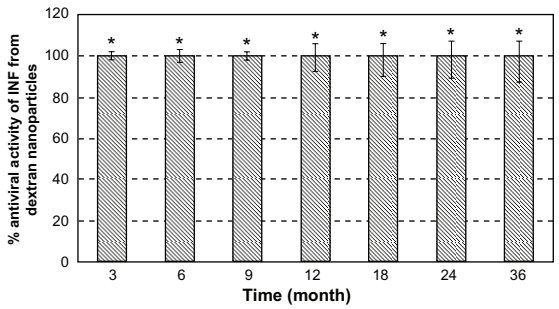

Figure 7 Stability of IFNα-2b–loaded dextran nanoparticles at 37°C (n = 5).
Note: *$P > 0.05$.

Conclusion

This work has outlined a straightforward means of high-efficiency loading of IFNα-2b dextran nanoparticles using a low temperature induced PEG aqueous phase/aqueous-phase emulsion method. The size of the dextran nanoparticles was able to be controlled by changing the temperature. The nanoscale-sized particles were easier to develop further different kinds of formulations with than microscale-sized particles. The nanoparticles were also able to preserve the high bioactivity of IFNα-2b during the fabrication and stock processes. Other biofriendly polymers could presumably replace dextran and PEG and provide a similar preparation method of a protein-loaded polysaccharide nanoscale-sized particle system. Under relatively mild conditions, the use of the low temperature induced PEG water phase/dextran water-phase emulsion method was favorable for the preservation of IFNα-2b integrity and biological functionality. This work advanced this method for the development of subunit vaccine and delivery devices for medically interesting

proteins and cell factors. The IFNα-2b loaded nanoparticles were easy to prepare in different formulations according to clinical needs.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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