International Journal of Pharmaceutics 407 (2011) 31–37

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Protamine modified metal ion–protein chelate microparticles for sustained release of interferon

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ARTICLE INFO

Article history:
Received 17 September 2010
Received in revised form
28 November 2010
Accepted 5 January 2011
Available online 8 January 2011

Keywords:
Interferon
Zinc
Protamine
Sustained release
In vitro release
Pharmacokinetics

ABSTRACT

This study focuses on extending the release period of zinc–protein chelate through protamine modification. Recombinant human interferon-α-2b (rhIFN), a highly pleiotropic cytokine with a short intrinsic pharmacokinetic half-life when injected subcutaneously (~2–6 h), was used as a model drug. Protamine modified zinc–rhIFN chelate microparticles were prepared by co-precipitating rhIFN with zinc and protamine. Introduction of protamine (2.5–20 mg/mL) into the chelation system had several prominent effects. First, percentage of chelated rhIFN was lowered (from >99% to ~90%); second, particle size was gradually increased (from ~0.45 μm to ~2 μm); last but important, it extended the release period of the chelate both *in vitro* (complete release was retarded from 8 h to 48 h) and *in vivo* ($t_{1/2}$ was prolonged from 4.5 h to 15.5 h and mean residence time from 9.4 h to 29.6 h). Size-exclusion liquid chromatography and cytopathic effect inhibition assay indicated rhIFN preserved its structural and functional integrity in these chelates.

1. Introduction

Metals are known to form bimolecular and bioinorganic interactions with proteins (Matthews et al., 2008). A certain protein presents different interaction manners towards different metal ions, including binding sites, chelation affinity and stability (Dienys et al., 2000). In pharmaceutical research, proteins are often precipitated into fine particles through chelation with bivalent metal ions, for example, Zn^{2+}. Such strategies formulate the soluble protein molecules into immobile and tight conjugates with metal ions, which can allow for sustained release of the therapeutic protein as carrier-free delivery system and thus avoid the laborious need of repetitive dosing (Gietz et al., 2000, 1998; Hallas et al., 1952).

Recombinant human interferon-α-2b (rhIFN), model protein in this study, is a highly pleiotropic cytokine in management of various neoplastic disorders and chronic viral diseases (Muss, 1987; Quesada et al., 1984; Samuel, 2001). It is of a short serum half-life when injected subcutaneously (~2–6 h) and frequent administration must be made to maintain the therapeutic efficiency. Several formulations with protracted effect have been developed, including biodegradable microspheres (Sánchez et al., 2003), multivesicular liposomes (Qiu et al., 2005) and molecular modification with polyethylene glycol (PEGylation) (Foser et al., 2003; Grace et al., 2001). During these formulations, the preparation of PLGA microspheres often incorporates conditions that denature and inactivate many proteins, such as elevated temperatures, surfactants and aqueous/organic solvent interfaces; also, the polymers still had to been dissolved in organic solvents (such as methylene chloride) which would affect the biological activity of the target protein (Lee et al., 1997). Other techniques either include complicated manufacturing processes which result in relatively low loadings of the active drug substances, or involve chemical modification and redesign of the molecules which lead to the creation of new molecular entities.

Alternatively, metal ion–protein chelation technology provides us another promising approach. The present study investigates the potential of using metal ion–rhIFN chelate microparticles for local sustained release application of rhIFN. Zinc ions were employed as chelating agent for facilitating microparticles formation. To extend the *in vitro* and *in vivo* release profiles of drug, protamine, a polycationic protein was employed as surface modifier. Physico-chemical characterization of the chelate particles with and without modification by protamine, including morphology, particle size, biological potency assay and *in vitro* release behavior, were compared. Moreover, *in vivo* serum pharmacokinetics of the zinc–rhIFN chelate microparticles were also investigated.

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doi:10.1016/j.ijpharm.2011.01.003

2. Materials and methods

2.1. Materials

Recombinant human interferon α-2b (rhIFN) from *Escherichia coli*, with a specific activity of 1.36×10^8 IU/mg, was provided by Huaxin High Biotechnology Inc. (Shanghai, China). Protamine sulfate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other reagents were of analytical grade.

2.2. Chelation of rhIFN with zinc ion

Chelation of rhIFN with zinc ion was achieved by mixing equal volume of rhIFN and zinc solution (pH 7.0). The mixture was kept at 4 °C overnight. The resulted suspension was centrifuged at 10,000 rpm/min for 5 min and the supernatant was removed for size-exclusion liquid chromatography (SEC-HPLC) analysis to determine the amount of free rhIFN. Chelation efficiency (C.E.) was calculated according to the following:

$$\text{C.E.}(\%) = \left(1 - \frac{W_{\text{free}}}{W_{\text{add}}}\right) \times 100$$

where W_{free} was the amount of free rhIFN in the supernatant, and W_{add} was the total amount of rhIFN added.

2.3. Modification of zinc–rhIFN chelate with protamine

Modification of zinc–rhIFN chelate with protamine was accomplished by co-precipitating of rhIFN with zinc and protamine. Briefly, protamine was dissolved in the zinc solution and mixed with rhIFN solution at a volume ratio of 1:1. The resultant mixture was also kept at 4 °C overnight.

2.4. Scanning electron microscopy (SEM)

Samples for morphological examination were gently washed with and dispersed in double-distilled water, and frozen at −20 °C overnight followed by lyophilization using a FD-1C-50 lyophilizer (Beijing, China). The lyophilized powder was loaded onto a metal stub with double sided tape and sprayed with gold vapor. Then the SEM analysis was carried out at 5 kV voltage on a Hitachi S-2400N scanning electron microscope.

2.5. Size and zeta potential analysis

Samples for sizes and zeta potential analysis were also washed with and then dispersed in double-distilled water before the analysis. The sizes and zeta potentials of the chelate particles were analyzed by a NICOMP™ 380 ZLS Zeta Potential/Particle Sizer (CA, USA).

2.6. High performance liquid chromatograph (HPLC)

The content determination of protamine in the chelates was performed by reverse-phase HPLC (RP-HPLC). The chromatographic system (Shimadzu, Japan) consists of a LC-10AT pump, a SPD-M10A VP UV–vis detector, a CTO-10AS VP column oven and a CLASS-VP Workstation. Chromatographic separation was performed on a WP 300 column (4.6 mm × 250 mm ID, Hanbon Sci.&Tech., China). The mobile phase was composed of 94% of phosphate buffer (pH 2.0) and 6% of acetonitrile. The column was eluted at the flow rate of 1.0 mL/min, and eluent was monitored at 214 nm.

Determination of rhIFN was performed by SEC-HPLC. The chromatographic system and separation were the same as above but with a different column (Protein-Pak 125, 300 mm × 7.8 mm ID, Waters, USA) and a different mobile phase (phosphate-buffered saline).

2.7. Cytopathic effect inhibition assay

To determine the biological potency retention of rhIFN after chelation with zinc, a cytopathic effect inhibition assay was performed. The vesicular stomatitis virus (VSV) was used to produce cytopathic effect on the human amnion WISH cells, as previously described by Rubinstein et al. (1981). WISH cells (3.5×10^5 cells/mL) were seeded in a 96-well microtiter and incubated with 4-fold serial dilutions of rhIFN samples for 24 h at 37 °C. After the virus diluent (100 CCID50) was added, the plates were incubated for 24 h until the cytopathic effect with 90% cell lysis was evident in the virus control wells. WISH cell viability was determined by measuring the absorbance of crystal violet-stained living cells in an ELISA plate. The relative potency of rhIFN was expressed as percentage of dilution multiples difference that shows 50% protection of VSV-induced WISH cells (ED_{50}) between the chelated and standard rhIFN samples.

2.8. In vitro release

The chelate particles (~70 μg) isolated from mother liquor were suspended in 200 μL Tris–HCl buffer (pH 7.4) containing 25 mM phosphate and incubated at 37 °C on a shaker (50 rpm/min). At set intervals, the entire suspensions were subjected to centrifugation at 5000 rpm/min for 5 min. The resultant pellets were re-suspended in equal volume of fresh release medium. The amount of rhIFN released in the supernatant was evaluated by SEC-HPLC analysis. The cumulative rhIFN percentage released from the chelates was calculated as the ratio of the amount of rhIFN released at time (t) to the initial amount used.

2.9. Animal test

Healthy rabbits weighing 2.0 ± 0.1 kg were provided by the pharmacological laboratory of our school. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Shenyang Pharmaceutical University, PR China. The animals were randomly assigned to three groups followed by the administration of soluble rhIFN, zinc–rhIFN chelate and protamine modified zinc–rhIFN chelate by subcutaneous injection at a single dose of ~1.5×10^7 IU. Blood samples were collected at determined time intervals followed by coagulation and centrifugation at 5000 rpm/min for 5 min. The resulting serum layers were separated and stored at −80 °C until ELISA analysis.

2.10. Pharmacokinetics and statistical analysis

The mean serum concentration profiles for each rhIFN formulation were constructed. The pharmacokinetics parameters of half-life ($t_{1/2}$), mean residence time (MRT) and the area under the concentration–time curve (AUC) were derived from the profiles using a noncompartmental model of WinNonlin® software package (Pharsight Corporation, USA).

3. Results and discussion

Polyelectrolytes can be used as surface modifiers for solid or liquid materials by means of electrostatic adsorption to form layered structures with unique properties (Antipov and Sukhorukov, 2004). In pharmaceuticals, drug loaded cores are usually coated with polyelectrolytes to form a protective envelope shielding the active ingredient from environmental interruption. In such a way,

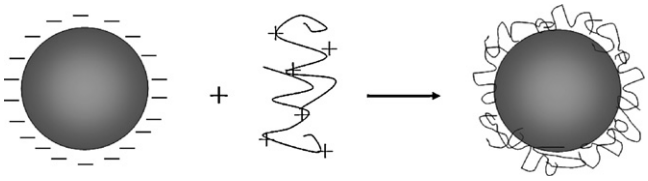

Fig. 1. Schematic illustration of fabrication of negatively charged zinc–rhIFN chelates by the polycationic protein, protamine.

drug delivery systems with controlled- or sustained-release functions can be prepared (Qiu et al., 2001; She et al., 2010).

We employed protamine, a polycationic protein, to fabricate the negatively charged zinc–rhIFN chelates (schematic illustration seen in Fig. 1.). The obtained protamine modified zinc–rhIFN chelate microparticles presented well-defined dissolution patterns both *in vitro* and *in vivo*. Protamine, first studied by Friedrich Miescher in 1868 (Ando et al., 1973), consists of strongly basic aliphatic and peptides rich in arginine group. These peptides are present in the sperm cell nuclei of fish with molecular masses ranging 4000–6000 Da (Kossel, 1929). Protamine and its salts, such as sulfate, are now well established as pharmaceutical excipients in insulin formulations (Nelson et al., 2009, 2001) and certain controlled-release formulations for antisense oligonucleotides (Junghans et al., 2005; Kratzer et al., 2007). Clinically, protamine sulfate is usually administered intravenously to reverse the large dose of heparin (Gillis, 1998). A concentration of 10 mg of protamine sulfate per mL is usually used.

3.1. Chelation of rhIFN with zinc ions in the presence of protamine

With or without protamine in the precipitation system, the chelation of rhIFN with zinc at neutral pH would both lead to the formation of a milky suspension. When inspected in the form of lyophilized powders by SEM, the chelates were displayed as small and grainy particles, seen in Fig. 2.

Introduction of protamine into chelation system composed of rhIFN and zinc brought down the chelation efficiency slightly. When protamine was added at 1 mg/mL, the chelation efficiency was lowered to ~94%; and when protamine was increased to 2.5 mg/mL, the chelation efficiency was down to ~90%. There were two plausible for this. First, as protamine was provided as sulfate salt, its addition would lower the pH of the precipitation system since sulfuric acid is a strong acid. Precipitation systems with lowered pH were unfavorable for the chelation of zinc ions and rhIFN. However, when the protamine solution was adjusted to pH 7.0, the

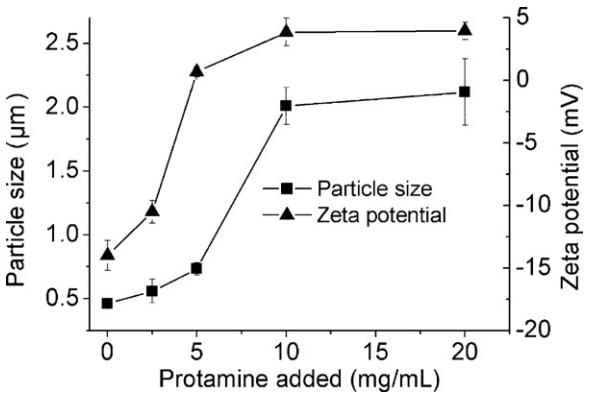

Fig. 3. Sizes (■) and zeta potentials (▲) of zinc–rhIFN chelates as a function of protamine added. All data are presented as means ± SD ($n = 3$).

reduction in chelation efficiency remained, suggesting there were some other underlying causes. As has been proved by Norrman et al. (2007), in protamine–zinc–insulin chelate, the positions at which protamine molecules bind to insulin molecules are close to those where zinc ions interact with insulin molecules. A similar interaction manner can be expected in protamine-modified zinc–rhIFN chelate. As a kind of Lewis acids, zinc ions can coordinate with glutamic acid residues of rhIFN, which can be thought as Lewis bases that give electron pairs. Therefore, the binding of protamine molecules to rhIFN molecules would block the interaction between zinc ions and rhIFN molecules. Interestingly in our study, when the concentration of protamine added was above 2.5 mg/mL (5–20 mg/mL), the percentage of the chelated rhIFN did not continue to decrease but remained almost the same as that with 2.5 mg/mL. This indicates that when added at 2.5 mg/mL, protamine was able to make saturated bondage with rhIFN molecules.

3.2. Particle size and zeta potential

Without protamine modification, the chelate particles were around 0.45 μm in diameter and were of negative charges (zeta potential was ~−14.0 mV). With the increase in amount of protamine added, the negative charges of the zinc–rhIFN chelate particles tended to be gradually neutralized. As the protamine concentration added increased from 2.5 mg/mL to 10 mg/mL, the zeta potential of the particles obtained were raised from ~−10.5 mV to ~4.0 mV (seen in Fig. 3). This neutralization also lowered the

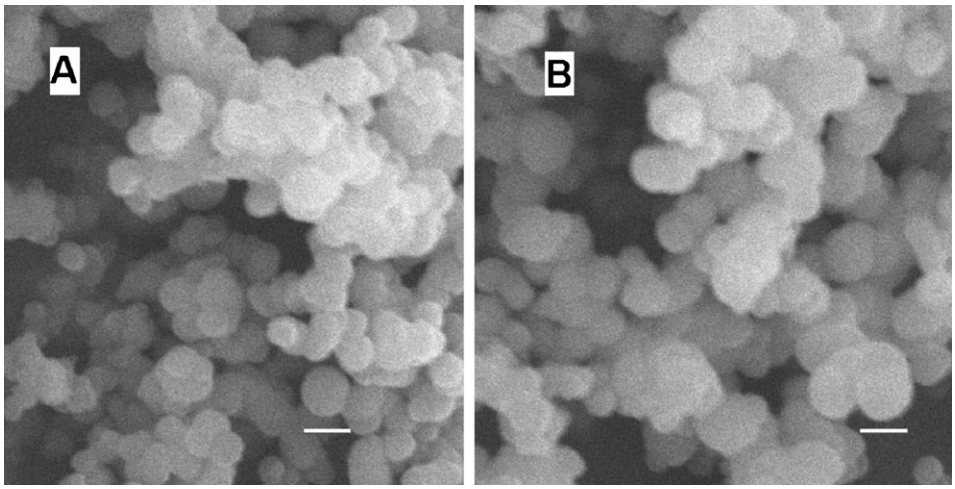

Fig. 2. SEM images of zinc–rhIFN chelate particles prepared without (A) and with protamine (5 mg/mL) (B) (Scale bar: 0.5 μm).

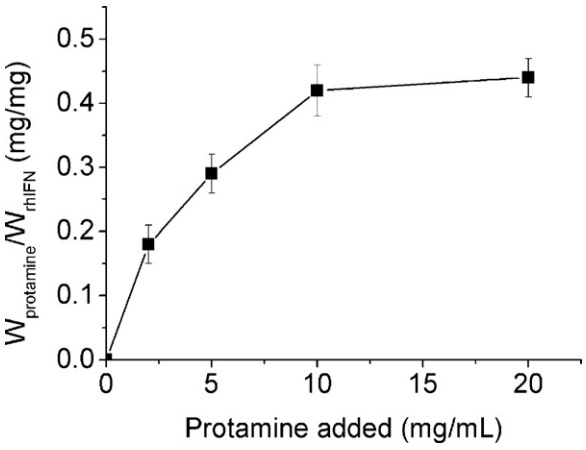

Fig. 4. Protamine content in the zinc–rhIFN chelates. All data are presented as means ± SD (n = 3).

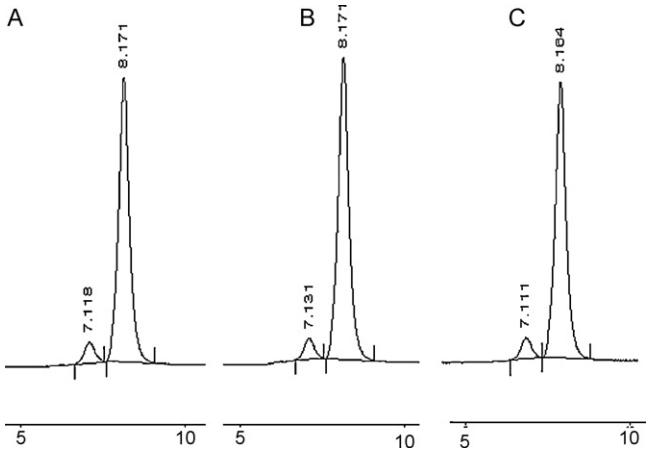

Fig. 5. SEC-HPLC chromatographs of rhIFN: (A) before chelation; (B) chelated with zinc and (C) with protamine modification. The chelate particles were dissociated in pH 7.4 Tirs–HCl buffer containing 25 mM EDTA before SEC-HPLC analysis.

electrostatic repulsive forces between the chelate particles. Therefore, with more protamine added, the chelate particles were prone to aggregate and greater particle sizes were rendered (Fig. 3). However, a further increment in protamine concentration (from 10 mg/mL to 20 mg/mL) did not bring down the zeta potential magnitude of the zinc–rhIFN particles, indicating the chelate particles were possibly fully covered on their surfaces by protamine molecules at 10 mg/mL. That the particles made from 10 mg/mL and 20 mg/mL protamine were of similar sizes (Fig. 3) also support this point. The micro sizes would make the particles prepared from 10 mg/mL and 20 mg/mL protamine tend to settle very quickly in water, during which the van der Waals attractive forces could lead the particles to be bound to each other strongly (i.e., cannot re-suspend) when the particles are close enough. One strategy to improve the suspension stability is to add polyethylene glycols (PEGs) with large molecular weight (>6000) into these systems (Yuan et al., 2007), which would not be stressed too much here.

The covering of protamine on the surface of zinc–rhIFN chelate particles can also be reflected by protamine content in the chelates indirectly. In this study, the content of protamine in the chelates was presented as mass ratio to rhIFN, seen in Fig. 4. The protamine content profile was of a similar pattern to the particle size/zeta potential profiles. Added at concentrations of 2.5–10 mg/mL, protamine interacted with rhIFN in an increasing manner and greater $W_{protamine}/W_{rhIFN}$ ratio was gradually rendered. However, further promoting the concentration (to 20 mg/mL) was only found to cause little increase in the protamine content in the chelates. This result was also supportive for the point that the chelate particles were possibly fully covered on their surfaces by protamine molecules at 10 mg/mL.

According to the results above, we can see that protamine could interact with rhIFN in two different ways during the chelation of rhIFN with zinc ions. First, protamine could structurally bind to rhIFN molecules at certain special locations. This binding interaction could be saturated as protamine concentration increased (from 1 mg/mL to 2.5 mg/mL). Second, protamine could also adsorb to the surface of zinc–rhIFN chelate particles through electrostatic attraction. A full coverage of the particle surface could be reached by raising the protamine concentrations from 5 mg/mL to 10 mg/mL.

3.3. Biological activity evaluation

Since the formation of chelate with zinc ions and further assembly into microparticles may lead proteins to change their native structure, the remained bioactivity needs to be identified. After dissociation by EDTA, the structural integrity of released protein was measured by SEC-HPLC. It was found that there were no detectable changes in the retention time and peak shape of rhIFN before and after formation into chelates with zinc (Fig. 5), which suggested that little chemical degradation of rhIFN occurred within the formulation process.

Cytopathic effect inhibition test was employed to assay the biological activity of rhIFN after being made into zinc chelates. While the anti-virus activity of the chelate without protamine was somewhat lowered (the percentage of cytopathic effect inhibition to the rhIFN before chelation was ~85%), the chelates prepared with protamine well maintained their antiviral activities (the percentages of cytopathic effect inhibition to the rhIFN before chelation were all >90%). These results show that the functional part of rhIFN was not affected during chelate formation with protamine. Together with the results of SEC-HPLC, it is indicated that both the structural and the functional integrity of rhIFN was relatively well preserved during the formation into chelate particles with zinc ions and succeeding modification by protamine.

3.4. In vitro release behaviors of zinc–rhIFN chelate microparticles

As described above, protamine could interact with rhIFN in the chelates through structural binding and electrostatic adsorption on the surface. It is believed that it is the internal binding and external coverage of protamine that enable rhIFN to be sustained-released from the chelates.

General *in vitro* release profiles are shown in Fig. 6A. Obviously, the addition of protamine into the chelation system reduced the initial burst (percentage of rhIFN released within 0.5 h) of the produced chelates (Fig. 6B), which should be attributed to the shield of the protamine layers on the surface of the chelates. Without protamine, more than 60% of protein was released from the chelate. When protamine was added at 2.5 mg/mL, the initial burst of the chelate was lessened to ~35%, suggesting the chelate particles were partially covered with protamine. As the protamine concentration rose to 10 mg/mL, the amount of rhIFN released from the chelate was decreased to ~15%. Doubling the protamine concentration (to 20 mg/mL) did not bring a further reduction in initial burst (Fig. 6B), indicating the chelate particles were fully covered with protamine at 10 mg/mL, which is consistent with the results of size and zeta potential analysis.

Moreover, involvement of protamine in the chelate modified the release kinetics of rhIFN and extended the overall releasing period.

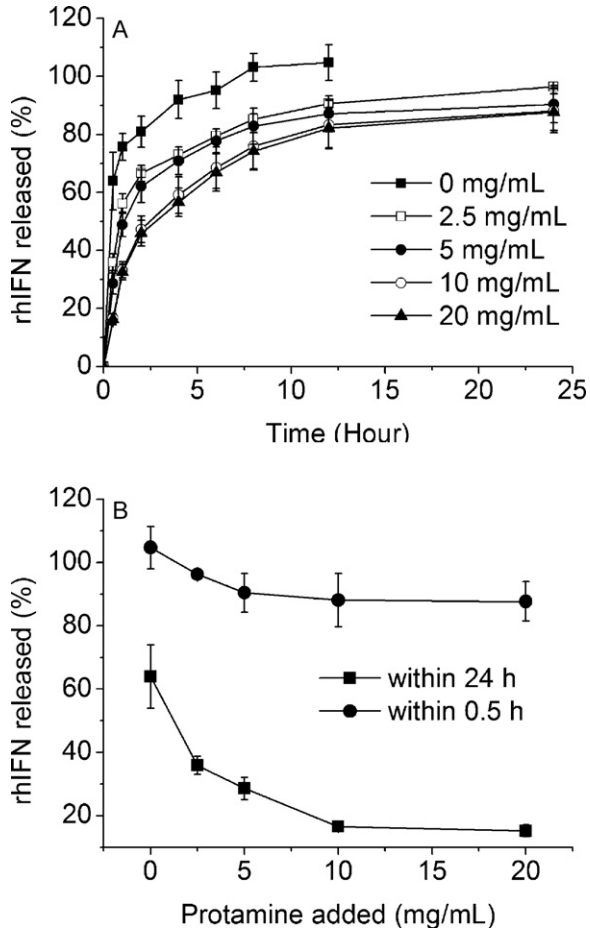

Fig. 6. In vitro release of zinc–rhIFN chelates modified with different concentrations of protamine: (A) general release profiles (protamine concentrations were: (■) 0 mg/mL; (□) 2.5 mg/mL; (●) 5 mg/mL; (○) 10 mg/mL and (▲) 20 mg/mL) and (B) rhIFN released from the chelates within 0.5 (●) and 24 h (■) as a function of protamine added. The dissolution medium was pH 7.4 Tris–HCl buffer containing 25 mM phosphate. All data are presented as means ± SD.

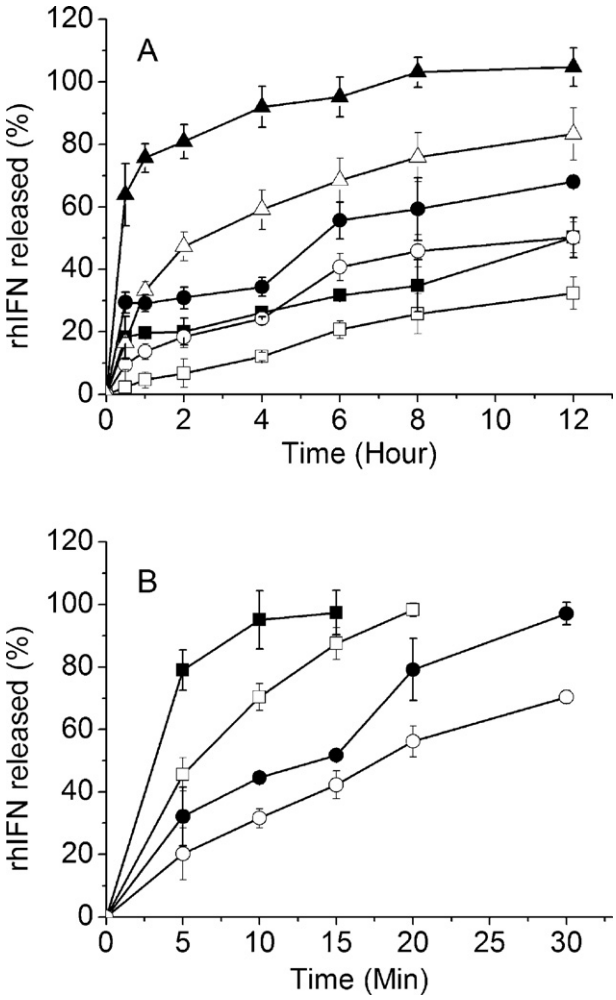

Fig. 7. In vitro release characters of zinc–rhIFN chelates prepared with (open symbols) and without (solid symbols) modification by protamine: (A) in dissolution buffers with different phosphate concentrations ((▲, △) 25 mM; (●, ○) 5 mM and (■, □) 1 mM) and (B) in dissolution buffers with 5 mM EDTA/citrate ((■, □) EDTA and (●, ○) citrate). All data are presented as means ± SD.

Without protamine modification, the complete release of protein from the chelate was reached at 8 h. However, after modification by protamine, the release of protein from the chelate was much slower (Fig. 6A) and the overall releasing period was extended to >24 h. In a more numeric way, only ~85% of the total protein was dissociated from the chelate in 24 h with 2.5 mg/mL protamine, and this percentage was down to ~80% with 10 and 20 mg/mL protamine (Fig. 6B). The complete dissociation of rhIFN from the chelates modified with 10 and 20 mg/mL protamine was retarded to 48 h.

Although protamine modification reduced the release kinetics of rhIFN and extended the overall releasing period, it did not alter the principal dissolution characteristics of the zinc–rhIFN chelates. Since zinc ions constitute essential chelating groups in the chelates, the introduction of ions that have greater chelation ability towards zinc ions than rhIFN into the dissolution medium would lead to the dissociation of zinc–rhIFN chelates, and the dissociation kinetics should be both affinity and concentration dependent. In our study, we found that the disintegration of zinc–rhIFN chelates was sensitive to both ionic species and concentrations added in the dissolution buffer, no matter whether the chelates were modified by protamine or not. With 5 mM EDTA, citrate or phosphate in the dissolution buffer, the chelates prepared without protamine modification would be completely dissociated within <10 min, ~30 min, or ~24 h, respectively. However, with the same species and concentrations of ions in the dissolution buffer, the corresponding complete dissociation time of protamine (10 mg/mL) modified chelates was much extended (Fig. 7). Because phosphate, citrate and EDTA have gradually greater chelation affinity towards zinc ions, the zinc–rhIFN chelates prepared showed a chelation rate-limiting dissolution pattern, which is of similarity to the dissolution patterns of zinc–insulin chelates (Prabhu et al., 2001). When the phosphate concentration was raised to 25 mM, the release of rhIFN was fastened; instead, with 1 mM phosphate in the dissolution medium, the release of rhIFN became slower, seen in Fig. 7A. Nevertheless, the chelate modified by protamine (10 mg/mL) still displayed much slower dissolution profiles (Fig. 7A)

3.5. *In vivo* pharmacokinetic study

To evaluate the sustained release ability of the developed formulations *in vivo*, the pharmacokinetic behaviors of the zinc–rhIFN chelate microparticles with or without protamine modification were investigated. Fig. 8 illustrates the serum rhIFN levels after subcutaneous injection of soluble and chelated rhIFN in health rabbits. Main pharmacokinetic parameters for serum rhIFN levels of each formulation are listed in Table 1. As seen in Fig. 8, when administrated subcutaneously at a single dose, the absorption of

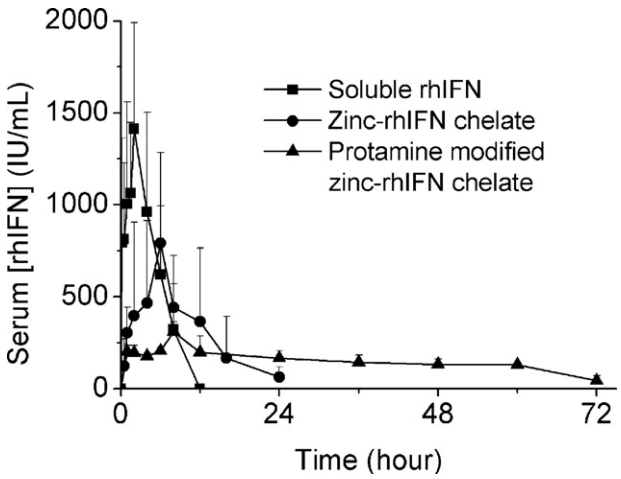

Fig. 8. Pharmacokinetic profiles of different rhIFN formulations in rabbits: (■) soluble rhIFN, (●) zinc–rhIFN chelate microparticles and (▲) protamine (10 mg/mL) modified zinc–rhIFN chelate microparticles. Each animal's predose (time = 0) rhIFN level was used for calculating elevation above baseline. Error bars are standard error of mean values (n = 4–5).

Table 1
Pharmacokinetic parameters for serum rhIFN levels (mean ± SD, n = 4–5).

rhIFN formulation	Soluble	Chelate	Protamine modified chelate
C_{max} (IU/mL)	1141 ± 580	773 ± 394	294 ± 55
T_{max} (h)	2.0 ± 0.0	6.0 ± 1.4	10.0 ± 2.3
$t_{1/2}$ (h)	2.5 ± 0.7	4.5 ± 0.8	15.5 ± 1.9
MRT (h)	4.4 ± 1.0	9.4 ± 2.5	29.6 ± 6.8
AUC_{0-t} (IU h/mL)	6946 ± 3803	6414 ± 3249	8623 ± 449

soluble rhIFN solution was rapid with a much higher C_{max} value of 1411 ± 580 IU/mL which were reached at 2 h after dosing. The mean residence time (MRT) of soluble rhIFN solution was 4.4 h and terminal half life ($t_{1/2}$) was 2.5 ± 0.7 h. Compared with the soluble rhIFN, zinc–rhIFN chelates (with or without protamine) provided prolonged PK profiles. Zinc–rhIFN chelate microparticles showed a much lower C_{max} value of 773 ± 394 IU/mL and a prolonged T_{max} of ∼6 h. Its MRT and $t_{1/2}$ were also extended to 9.4 ± 2.5 h and 4.5 ± 0.8 h, respectively. More significantly, the C_{max} of protamine modified zinc–rhIFN chelate microparticles was lowered to 294 ± 55 IU/mL and T_{max} was retarded to ∼10.0 h. After its peak the rhIFN level began to descend slowly to baseline at ∼72 h, with MRT and $t_{1/2}$ being 29.6 ± 6.8 h and 15.5 ± 1.9 h, respectively, displaying a distinctively long-acting feature. Moreover, the low burst release from the administration of protamine-modified zinc–rhIFN chelate microparticles avoided potentially problematic serum levels and thus provided safety.

4. Conclusion

Formulating rhIFN into chelate with zinc could prolong its release profiles both *in vitro* and *in vivo*. Addition of protamine into the chelation system could obtain protamine-modified zinc–rhIFN chelate microparticles with even prolonged release profiles for rhIFN, which can be applied to chelation systems of zinc with other proteins. Since amino acids (Dolinska, 2001) and peptides (Dolinska and Ryszka, 2003; Qian et al., 2009; Ryszka and Dolinska, 2003a,b) are also of the properties to form chelates with zinc, the tactic involvement of protamine into the zinc–protein chelates elongates the release period of the target protein that can also be applicable to amino acid and peptide formulations.

Acknowledgements

The authors wish to thank the National Natural Science Foundation of China (No. 30701057) and Scientific research plan projects of Liaoning Education Department (No. 2009A806) for financial support.

References

Ando, T., Yamasaki, K., Suzuki, K., 1973. Protamines: Isolation, Characterisation, Structure and Function. Springer, Berline.
Antipov, A.A., Sukhorukov, G.B., 2004. Polyelectrolyte multilayer capsules as vehicles with tunable permeability. Adv. Colloid Interface Sci. 111, 49–61.
Dienys, G., Sereikaite, J., Luksa, V., Jarutiene, O., Mistiniene, E., Bumelis, V.A., 2000. Dimerization of human growth hormone in the presence of metal ions. Bioconjugate Chem. 11, 646–651.
Dolinska, B., 2001. The properties of solid Zn(II)-amino acid complexes in the form of suspensions. Farmaco 56, 737–740.
Dolinska, B., Ryszka, F., 2003. Preparation and properties of selected Zn(II)-peptide complexes in suspension. Farmaco 58, 1131–1136.
Foser, S., Schacher, A., Weyer, K.A., Brugger, D., Dietel, E., Marti, S., Schreitmüller, T., 2003. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α-2a (PEGASYS). Protein Expr. Purif. 30, 78–87.
Gietz, U., Arvinte, T., Haner, M., Aebi, U., Merkle, H.P., 2000. Formulation of sustained release aqueous Zn-hirudin suspensions. Eur. J. Pharm. Sci. 11, 33–41.
Gietz, U., Arvinte, T., Mader, E., Oroszlan, P., Merkle, H.P., 1998. Sustained release of injectable zinc-recombinant hirudin suspensions: development and validation of in vitro release model. Eur. J. Pharm. Biopharm. 45, 259–264.
Gillis, M., 1998. CPS Compendium of Pharmaceuticals and Specialties, 33rd ed. Canadian Pharmacists Association, Ottawa.
Grace, M., Youngster, S., Gitlin, G., Sydor, W., Xie, L., Westreich, L., Jacobs, S., Brassard, D., Bausch, J., Bordens, R., 2001. Structural and biologic characterization of pegylated recombinant IFN-alpha2b. J. Interferon Cytokine Res., 21.
Hallas, M., Oller, K., Petersen, K., Schlichtkrull, J., 1952. Crystalline and amorphous insulin-zinc compounds with prolonged action. Science 116, 394–398.
Junghans, M., Loitsch, S.M., Steiniger, S.C., Kreuter, J., Zimmer, A., 2005. Cationic lipid–protamine–DNA (LPD) complexes for delivery of antisense c-myc oligonucleotides. Eur. J. Pharm. Biopharm. 60, 287–294.
Kossel, A., 1929. Protamine und Histone. Deuticker Verlag, Leipzig.
Kratzer, I., Wernig, K., Panzenboeck, U., Bernhart, E., Reicher, H., Wronski, R., Windisch, M., Hammer, A., Malle, E., Zimmer, A., Sattler, W., 2007. Apolipoprotein A-I coating of protamine-oligonucleotide nanoparticles increases particle uptake and transcytosis in an in vitro model of the blood–brain barrier. J. Control. Release 117, 301–311.
Lee, H.J., Riley, G., Johnson, O., Cleland, J.L., Kim, N., Charnis, M., Bailey, L., Duenas, E., Shahzamani, A., Marian, M., Jones, A.J., Putney, S.D., 1997. In vivo characterization of sustained-release formulations of human growth hormone. J. Pharmacol. Exp. Ther. 281, 1431–1439.
Matthews, J.M., Loughlin, F.E., Mackay, J.P., 2008. Designed metal-binding sites in biomolecular and bioinorganic interactions. Curr. Opin. Struct. Biol. 18, 484–490.
Muss, H.B., 1987. Interferon therapy for renal cell carcinoma. Semin. Oncol. 14, 36–42.
Nelson, R.W., Henley, K., Cole, C., 2009. Field safety and efficacy of protamine zinc recombinant human insulin for treatment of diabetes mellitus in cats. J. Vet. Intern. Med. 23, 787–793.
Nelson, R.W., Lynn, R.C., Wagner-Mann, C.C., Michels, G.M., 2001. Efficacy of protamine zinc insulin for treatment of diabetes mellitus in cats. J. Am. Vet. Med. Assoc. 218, 38–42.
Norrman, M., Hubalek, F., Schluckebier, G., 2007. Structural characterization of insulin NPH formulations. Eur. J. Pharm. Sci. 30, 414–423.
Prabhu, S., Jacknowitz, A.I., Stout, P.J., 2001. A study of factors controlling dissolution kinetics of zinc complexed protein suspensions in various ionic species. Int. J. Pharm. 217, 71–78.
Qian, F., Ni, N., Burton, L.S., Wang, Y.F., Desikan, S., Hussain, M., Smith, R.L., 2009. Sustained release subcutaneous delivery of BMS-686117, a GLP-1 receptor peptide agonist, via a zinc adduct. Int. J. Pharm. 374, 46–52.
Qiu, J., Wei, X.H., Geng, F., Liu, R., Zhang, J.W., Xu, Y.H., 2005. Multivesicular liposome formulations for the sustained delivery of interferon alpha-2b. Acta Pharmacol. Sin. 26, 1395–1401.
Qiu, X., Leporatti, S., Donath, E., Mohwald, H., 2001. Studies on the drug release properties of polysaccharide multilayers encapsulated ibuprofen microparticles. Langmuir 17, 5375–5380.
Quesada, J.R., Reuben, J., Manning, J.T., Hersh, E.M., Gutterman, J.U., 1984. Alpha interferon for induction of remission in hairy-cell leukemia. N. Engl. J. Med. 310, 15–18.
Rubinstein, S., Familletti, P.C., Pestka, S., 1981. Convenient assay for interferons. J. Virol. 37, 755–758.
Ryszka, F., Dolinska, B., 2003a. Pulsatile and moderated release of dalareline from Zn(II) complexes in the form of suspension. Boll. Chim. Farm. 142, 10–13.

Ryszka, F., Dolinska, B., 2003b. Pulsatile and sustained peptides release from Zn(II) suspensions. Boll. Chim. Farm. 142, 319–323.
Sánchez, A., Tobío, M., González, L., Fabra, A., Alonso, M.J., 2003. Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha. Eur. J. Pharm. Sci. 18, 221–229.
Samuel, C.E., 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. 14, 778–809.
She, Z., Antipina, M.N., Li, J., Sukhorukov, G.B., 2010. Mechanism of protein release from polyelectrolyte multilayer microcapsules. Biomacromolecules 11, 1241–1247.
Yuan, W., Wu, F., Geng, Y., Xu, S., Jin, T., 2007. An effective approach to prepare uniform protein–Zn^{2+} nanoparticles under mild conditions. Nanotechnology 18, 145601.