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ORIGINAL ARTICLE

A morphological screening of protein crystals for interferon delivery by metal ion-chelate technology

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Abstract

Objective: This study presents a preliminary exploration on extending the half-life of therapeutic proteins by crystallization strategy without new molecular entities generation. *Methods*: Recombinant human interferon (rhIFN) α-2b, a model protein drug in this case, was crystallized using a hanging-drop vapor diffusion method. A novel chelating technique with metal ions was employed to promote crystals formation. *Results*: The effects of key factors such as seeding protein concentration, pH of the hanging drop, ionic strength of the equilibration solution, and precipitants were investigated. Size-exclusion liquid chromatography, antiviral activity determination, and enzyme-linked immunosorbent assay indicated that both the molecular integrity and biological potency of rhIFN were not significantly affected by crystallization process. In addition, the in vitro release behavior of rhIFN from crystal lattice was characterized by an initial fast release, followed by a sustained release up to 48 hour. *Conclusion*: The work described here suggested an exciting possibility of therapeutic protein crystals as a long-acting formulation.

Key words: *Bioactivity; crystallization; interferon; morphology; sustained release*

Introduction

The advent of recombinant molecular biology in the 1970s and constant improvements in expression methods in the subsequent two decades have led to great availability of biomacromolecules, many of which have been proved to be quite effective in treating cancerous, inflammatory, cardiovascular, respiratory, and infectious diseases. However, their chemical instability, rapid clearance from systemic circulation, susceptibility to enzymatic degradation, and thus short half-life bring huge obstacles for them to be fully used as therapeutic agents[1,2]. Therefore, much attention has been paid to the development of extended release formulation of therapeutic proteins these years. Among the techniques used for extending the half-life of proteins are PEGylation[3], fusion protein technology[4], microcapsulation by biodegradable polymers[5,6], and so on. All these techniques either involve chemical modification and redesign of the molecules and thus create new molecular entities or include complicated and expensive manufacturing processes and result in relatively low loadings of the active drug substances. Fortunately, protein crystallization technology provides us another promising approach. Compared to its amorphous or lyophilized forms, therapeutic proteins are aligned in the crystals by a highly ordered manner and hence are protected in the lattice structure during processing, upon storage, and during drug release[7]. Moreover, protein crystals can also allow sustained release of the therapeutic agents as carrier-free delivery system, thus avoiding the labor- and cost-intensive need of repetitive dosing[8]. Because of great challenges in combination of protein crystallization engineering and pharmaceutical science, so far quite a few therapeutic proteins have been crystallized and only one product, insulin, that is produced has been administered in crystalline form[9].

Interferon is of great potent in management of various neoplastic disorders and chronic diseases including chronic myelogenous leukemia, malignant melanoma, and hepatitis[10,11]. In hospital or clinical settings, because

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of its short serum half-life when injected subcutaneously (about 2–6 hours) or intravenously (several minutes), frequent administration of recombinant human interferon (rhIFN) α-2b dose must be made to maintain the therapeutic efficiency. Several formulations have been developed to achieve the goal of protracted effect including biodegradable microspheres[12,13], multivesicular liposomes[14,15], and molecular modification with polyethylene glycol (PEG) (PEGylation)[16], among which PEGylation seems to be most successful and several PEGylated rhIFN products such as Pegintron™ and Pegasys™ are already on the market. The half-life of the PEGylated protein is 40 hours, thus it only needs to be administered once a week for similar therapeutic effects[17]. However, the chemical conjugation process of PEGylation is rather complex and involves generating new molecular entities, in which safety evaluation is a long process. In addition, it has been suggested that the bioactivity of the protein may be affected to some extent[18].

This work investigates the potential of using protein crystals for local sustained release application in light of this new progress. A preliminary exploration on the crystallization of rhIFN was carried out by the hanging-drop vapor diffusion method (Figure 1). Zinc ions were used as chelating agent for facilitating crystals formation. The conditions for the production of rhIFN crystals, including seeding protein concentration, pH of the hanging drop, ionic strength of the equilibration solution, and some precipitants, were discussed. In addition, antiviral activity and immunological activity of the protein after crystallization were evaluated. Moreover, the in vitro release behaviors of rhIFN from crystal lattice were also investigated.

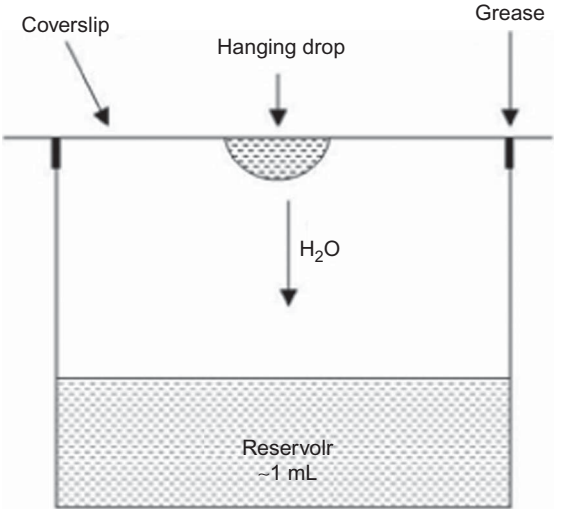

Figure 1. Schematic representation of hanging-drop vapor diffusion method for rhIFN crystallization.

Materials and methods

Materials

The rhIFN α-2b from *Escherichia coli*, with a specific activity of 1.36×10^8 IU/mg of protein, was purchased from Huaxin High Biotechnology Inc. (Shanghai, China). Vesicular stomatitis virus (VSV) and human amnion WISH cells were kindly provided by Liaoning Satellite Biotechnology Products Research Institute (Liaoning, China). PEG with average molecular weight of 2000, 4000, and 6000 were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ethylenediaminetetraacetic acid disodium (EDTA-Na) salt was obtained from Chemicals and Reagents Company (Guangzhou, China). All other reagents were of analytical grade.

Methods

Preparation of rhIFN crystals

Crystallization of rhIFN was performed using the hanging-drop vapor diffusion method in a 24-well Linbro plate (Linbro, Hamden, CT, USA)[19]. Briefly, 10 µL droplets containing 20 mg/mL of protein in 75 mM zinc acetate and 1.5 M sodium acetate, pH 5.5, were hung from siliconized coverslips inverted on Linbro tissue culture plates. These droplets were equilibrated against 1 mL of 150 mM zinc acetate and 1.5 M sodium acetate, pH 5.5, at 4°C until crystals occur. To determine the average size and morphology, crystals were photographed with a DM-BA400-B light microscope (Motic, Xiamen, China).

SEC-HPLC characterization of rhIFN crystals

Determination of rhIFN was performed by a size-exclusion liquid chromatography (SEC-HPLC). The chromatographic system (Shimadzu, Kyoto, Japan) consisted of an LC-10AT pump, an SPD-M10A VP UV–Vis detector, a CTO-10AS VP column oven, and a CLASS-VP Workstation. Chromatographic separation was performed on a Protein-Pak 125 column (300 × 7.8 mm ID, Waters, Milford, MA, USA). The column was eluted with phosphate-buffered saline (PBS) at a flow rate of 1.0 mL/min, and the eluant was monitored at 214 nm.

Antiviral activity

Antiviral activity of crystallized rhIFN was determined by inhibition of the cytopathic effect produced by VSV with human amnion WISH cells as previously described[20]. WISH cells (3.5 × 105 cells/mL) were seeded in a 96-well microtiter and incubated with four-fold serial dilutions of rhIFN samples (1000 IU/mL) for 24 hours at 37°C. After the virus diluent (100 CCID50) was added, the plates were incubated for 24 hours 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 enzyme-linked immunosorbent assay (ELISA) plate. The relative potency of crystallized rhIFN was expressed as percentage of dilution multiples difference that shows 50% protection of VSV-induced WISH cells (ED_{50}) between the crystallized and standard rhIFN samples.

Enzyme-linked immunosorbent assay

The immune activity of rhIFN after crystallization was quantified by a sandwich-ELISA technique[21]. In short, 96-polyvinyl well plates were coated with polyclonal antibodies against rhIFN diluted in PBS overnight at 4°C. After being blocked in 0.1% bovine serum albumin solution in PBS for 1 hour at 37°C, the plates were washed thrice with washing buffer (0.05%, v/v; Tween 20 in PBS). The wells were then loaded with a series dilution of rhIFN samples and incubated for 1 hour at 37°C. After they were washed thrice, mouse monoclonal anti-rhIFN antibodies conjugated with horseradish peroxidase (1:100 dilution in PBS) were added and incubated for 45 minutes at 37°C. Washed with washing buffer thrice again, the bound enzyme conjugate was detected by adding chromogenic substrates (2, 2'-Azino-di (3-ethylbenzthiazoline-6-sulforic acid) (ABTS), 100 μL per well) to the wells. The resulting color reaction was measured at 405 nm using a Synergy HT ELISA spectrophotometer (Bio-tek, Winooski, VT, USA).

In vitro release of rhIFN crystals

In vitro drug release of rhIFN from the crystals matrix was performed in PBS (pH 7.4) at 37°C with continuous orbital mixing (50 rpm/min). At appropriate intervals, the entire suspensions were subjected to centrifugation at 3800 × *g*/min for 10 minutes. The pellets were resuspended 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 crystal matrix was calculated as the ratio of the amount of rhIFN released at time (t) to the initial amount used.

Results and discussion

Crystallization of small molecular therapeutic substances has been used as conventional pharmaceutical technology for decades[22,23]. The main advantages include better stability, avoidance of chemical degradation, and varied dissolution characteristics that allow better control over bioavailability. These advantages can also be envisioned for therapeutic protein crystals. However, protein crystals have not received as much attention as small chemicals because of great challenges in biomacromolecular crystallization, which has been often called the 'bottleneck' in X-ray structural determination[24]. Recent advances, however, have alleviated many of the obstacles. In this work, macromolecular crystallization aided by metal ions as chelate ligand was used for developing protein crystals into drug formulation.

The zinc ion plays an important role in forming rhIFN crystals. In this study, it was found that all the efforts made to get crystals were in total vain if the zinc ions were not included into the equilibration system. This founding was also convinced by Radhakrishnan et al., who have shown exactly how the zinc ion works in rhIFN crystals through X-ray crystallography[25]. Through interacting with glutamic acid residues of rhIFN, a distorted tetrahedral zinc-coordination sphere is formed between two crystallographic symmetric units of rhIFN crystals, with each unit composed of three dimers of rhIFN molecules. It is, therefore, the involvement of zinc ion that endows the rhIFN crystal crystallographic integrity.

Effect of concentration of zinc ions

Considering the consistency with buffer ions, zinc acetate was selected as chelating agent for crystals formation. With 1.5 M acetate buffer (pH 5.5) and rhIFN concentration of 20 mg/mL, the hanging drop was allowed to equilibrate against reservoir solution under 4°C for 6 days. Crystal occurrence was observed in all the hanging drops containing three tested concentrations (i.e., 25, 50, and 75 mM) of zinc acetate. As seen in Figure 2, an increase in the concentration of zinc acetate led to an increase in the amounts and spherical degree of crystals formed. The crystals that came from 25 mM zinc acetate showed an irregular shape with mean diameters of about 100–200 μm, whereas both crystals from 50 and 75 mM zinc acetate presented a near-ellipsoidal shape and uniform size distribution of 70–100 and 25–50 μm, respectively.

Apart from being a chelation group in rhIFN crystals, residual zinc acetate can bind bulk water as a hydration layer near the surface of rhIFN. In this way, namely 'salting-out', zinc ions can compete with rhIFN molecules for water to make proteins partially dehydrated. Consequently, rhIFN molecules prefer to fill their exposed surfaces with other rhIFN molecules which promote their interactions[26]. Thus, in a certain range, an increase in the concentration of zinc acetate facilitated the formation of crystals and brought massive crystals in smaller size because of limited space and high rate of nuclei formation.

Effects of seeding concentration of proteins

To investigate the initial concentration of rhIFN on crystals formation, two concentrations of rhIFN (20 and

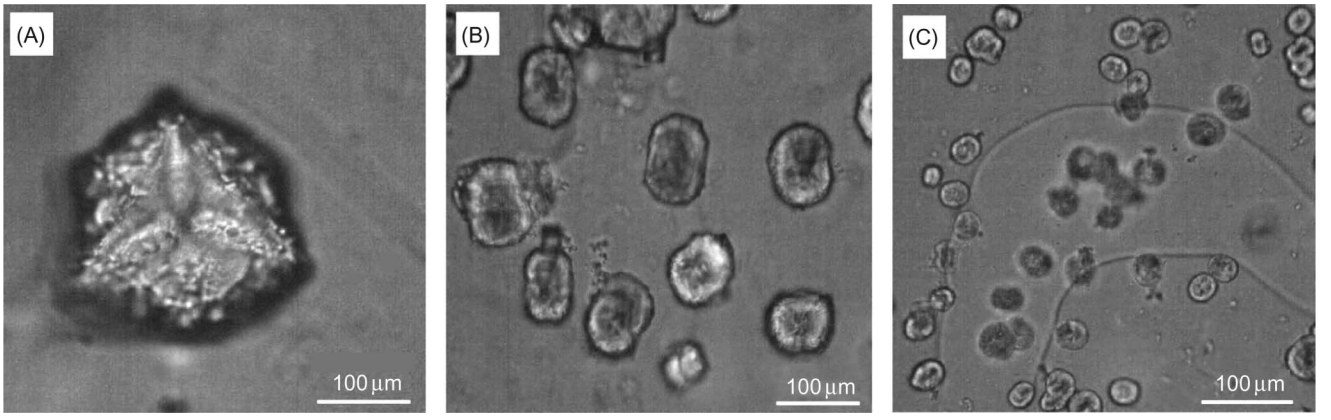

Figure 2. Different crystal morphologies were obtained with (A) 25, (B) 50, and (C) 75 mM of zinc acetate.

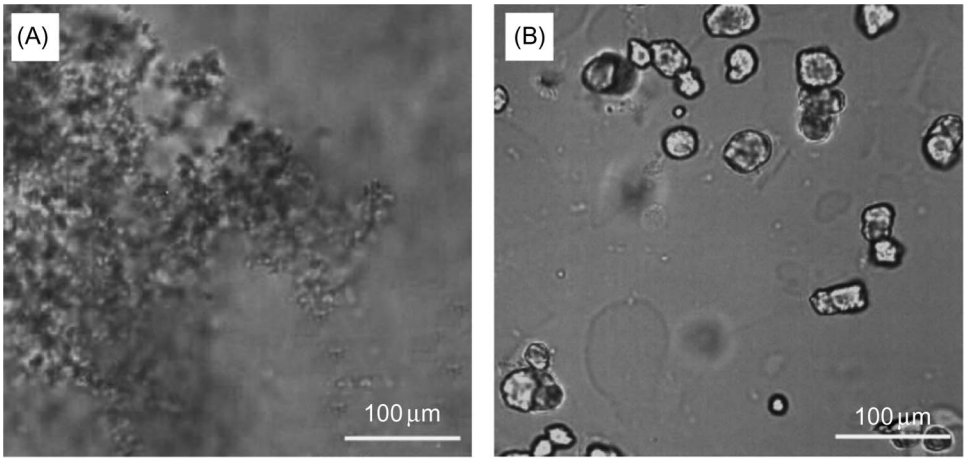

Figure 3. Different crystal morphologies were obtained with (A) 20 and (B) 40 mg/mL of seeding protein concentration.

40 mg/mL) were used for the crystallization with pH 5.5, 1.5 M acetate buffer solution containing 75 mM zinc acetate at 4°C. From Figure 3, one can tell that hanging drops with 40 mg/mL of rhIFN were all full of amorphous precipitates, which indicated that the supersaturation degree was too high for crystals formation. However, with the protein concentration decreased to 20 mg/mL, massive crystals with cubic shape can be observed in the hanging drops.

Crystal growth generally starts at solute concentrations sufficient for nucleation to occur and continues at concentrations beneath the nucleation threshold. The growth of crystals from nuclei is also strongly influenced by diffusion and convection effects. As with nucleation, increased protein concentration results in increased growth rates. However, random precipitation will occur unavoidably if the growth rate of nuclei is too high, which is often the result of high initial protein concentration[27].

Effects of ionic strength in buffer solution

The influence of ionic strength of salts on rhIFN crystallization was investigated by changing the concentration of acetate buffer in the reservoir solutions, while keeping all other conditions constant as pH 5.5, 20 mg/mL protein and 75 mM zinc acetate at 4°C. As shown in Figure 4, the hanging drop equilibrating against reservoir solution of 0.5 M acetate buffer gave amorphous precipitates, whereas cubic (Figure 4B) or spherical (Figure 4C) crystals for 1.0 and 1.5 M acetate buffer.

It was known that two effects, namely 'salting-in' and 'salting-out', can affect the solubility of proteins[28]. When at low salt concentration, the solubility of the protein increases as the ionic strength increases, which is referred to as 'salting-in'. During this investigation, as the concentration of acetate buffer raised from 0.5 to 1.0 M or 1.5 M, the occurrence of hanging drops was changed from random precipitation to full of crystals, showing

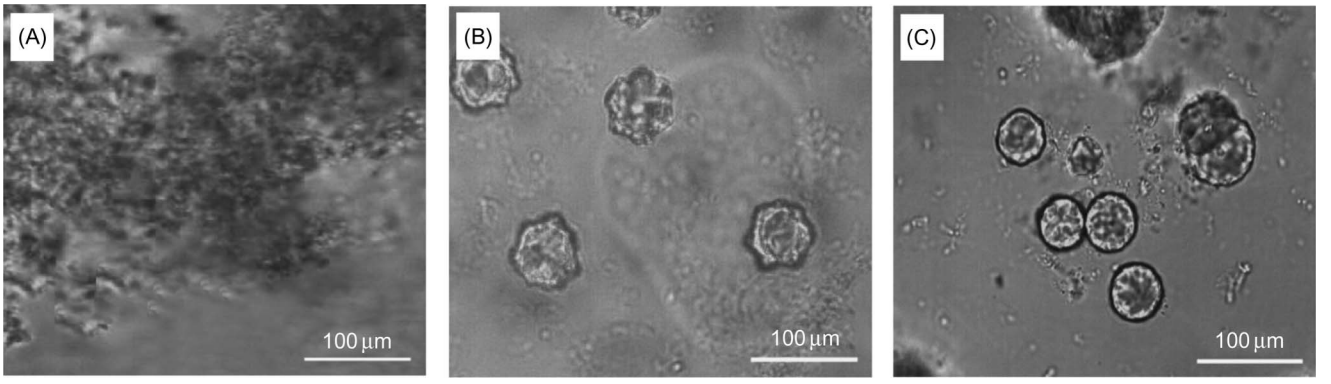

Figure 4. Different crystal morphologies were obtained with (A) 0.5, (B) 1.0, and (C) 1.5 mM of acetate buffer.

the solubility of protein was promoted by the increased ionic strengths or in other words exhibiting the so-called 'salting-in' phenomenon. Improving the protein solubility moderately by increasing the ionic strength in buffer solution can adjust the saturated state of the system and avoiding random precipitation.

Effects of pH values in buffer solution

Allowing the hanging drops to equilibrate against reservoir solution of different pH values and keeping all other conditions constant as 20 mg/mL protein and 75 mM zinc acetate in 1.5 M acetate buffer solution, occurrences in drops are shown in Figure 5. It was observed that drops with pH 5.5, 6.0, and 6.5 all gave crystals among which drops with pH 6.0 and 6.5 are accompanied with several white granules and a few precipitates separately. Amorphous precipitates were found in drops with pH 7.0.

Adjusting pH values in the equilibration systems will affect the electronic properties of the protein molecules and thus change the solubility and the supersaturation degree. It is well known that crystallization is most likely to occur in the pH range near the isoelectric point (pI) of the protein, at which the solubility of target protein is limited and supersaturation is easy to achieve[29]. Normally, the isoelectric point of rhIFN is 5.5–6.8[30], which means there is a greater likelihood of rhIFN crystallization to occur within this pH range. The finding of precipitates in the drops with pH 6.5 and 7.0 suggested that the solubility of rhIFN is lowest in this range.

Effects of precipitants

To promote crystals formation, acetone and PEG were used as crystallization auxiliary. As seen in Figure 6, addition of both 10% and 15% (v/v) acetone resulted in a mixture of large crystals and massive tiny precipitates, which indicated too strong a power for acetone in creating supersaturation.

The effects of PEG addition with various average molecular weights (Mw 2000, Mw 4000, and Mw 6000)

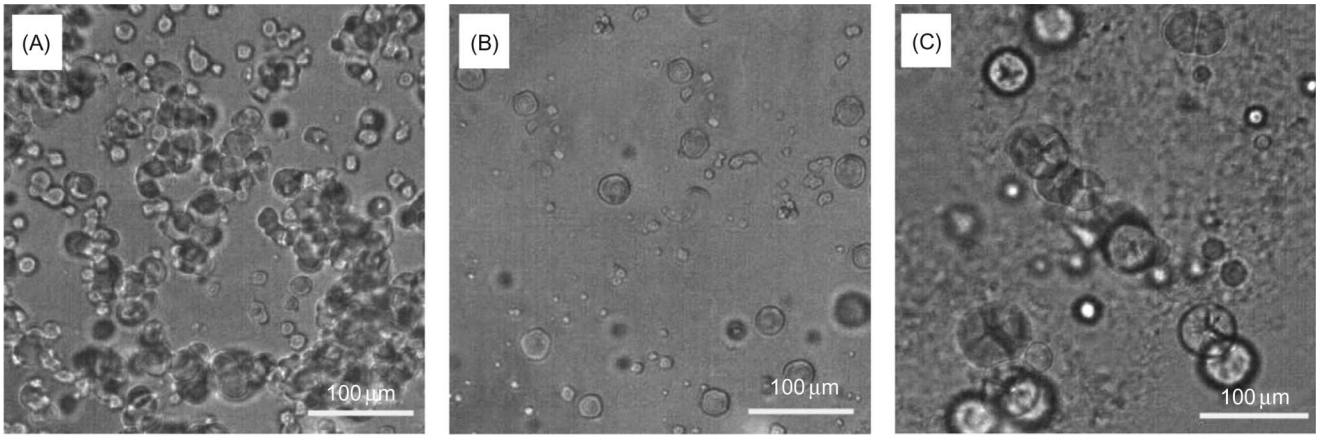

Figure 5. Different crystal morphologies were obtained with (A) pH 5.5, (B) pH 6.0, and (C) pH 6.5 in buffer solution.

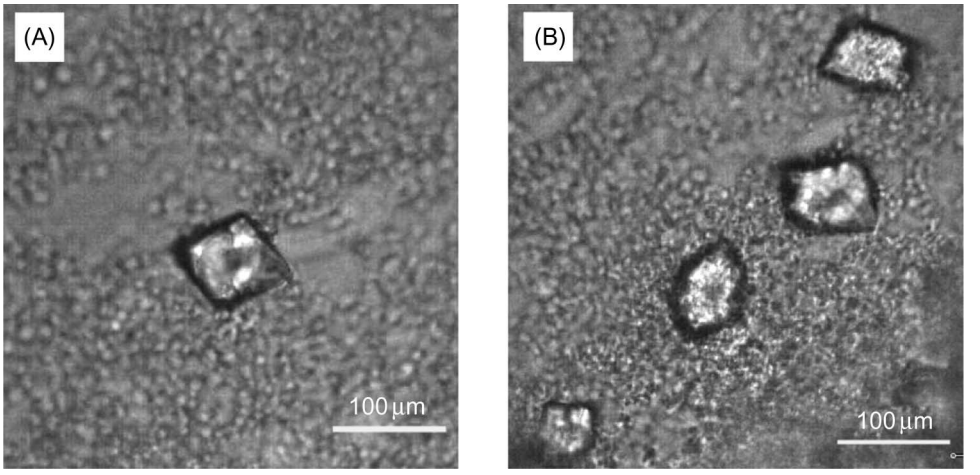

Figure 6. Different crystal morphologies were obtained with (A) 10% and (B) 15% of acetone.

and concentrations (50 and 100 mg/mL) on crystals formation were investigated and the happenings in hanging drops are shown in Figure 7. Compared with that from PEG 2000, the cubic crystals produced from PEG 4000 were uniform and small in size. However, minor crystals accompanied with a lot of precipitates could be found in hanging drops with PEG 6000.

Nucleation is a very slow process and can take a lot of time because of this high-energy barrier to cross. If the supersaturation is too low (in the metastable state), it would correspond to a very slow attainment of nucleation, and crystals might not be formed within a reasonable length of time. Higher supersaturation can be induced using a variety of precipitants. Precipitants work by weakening the interaction between protein molecules and water molecules whereas strengthening the interaction between protein molecules to benefit the formation of crystals[31]. Precipitants generally fall into four broad categories: salts, organic solvents, polymers, and nonvolatile organic compounds. The first two classes are typified by ammonium sulfate and ethyl alcohol, respectively, and higher polymers such as PEG 4000 are characteristic of the third. Compounds such as methylpentanediol and PEGs of molecular weight less than about 1000 are listed in the fourth category[32]. Strictly speaking, buffer salts and zinc acetate could also work as precipitants in this study. Acetone is a simple organic that is often used in producing protein crystals. It can reduce the dielectric constant of the solutes to weaken both their electrostatic repulsion and polarity and therefore increase the attraction between the protein molecules[33]. PEGs, most popular long-chain polymers used in macromolecule crystallization, can bind to the water molecules and produce volume exclusion effects that induce separation of macromolecules from solutions. In general, the higher the molecular weight of PEG is, the more efficient it decreases the solubility of the proteins[33]. Compared to PEG with low molecular weight of 1000 and 4000, PEG 6000 can bind to the surface of proteins and dehydrate them efficiently because of its longer linear chains, which usually lead to a precipitation occurrence.

Characterization of rhIFN crystals

According to the results of the above investigations, crystals with satisfactory size and shape were obtained at the following optimal conditions: with 75 mM zinc acetate addition, the hanging drop containing 20 mg/mL of rhIFN was allowed to equilibrate against reservoir solution with 1.5 M acetate buffer (pH 5.5) under 4°C. The crystals were recovered for the following characterizations.

After accelerated release from crystals by EDTA, the 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 crystallization and both major fractions eluted at 8.15 minutes were larger than 95% (seen in Figure 8), which suggested that low level of chemical degradation of rhIFN occurred within the formulation.

The biochemical function and activity of proteins is intimately associated with their three-dimensional structure. Comparing with the native protein, the formation of tight, geometrically precise intermolecular contacts of protein in crystal lattices may lead to a change in intermolecular interaction and surface properties of the protein, which may disturb protein receptor interactions and affect some of the protein's in vitro biological activities. After being dissociated by EDTA, the biological activity of the released rhIFN was assayed by cytopathic effect inhibition test. On an average, the

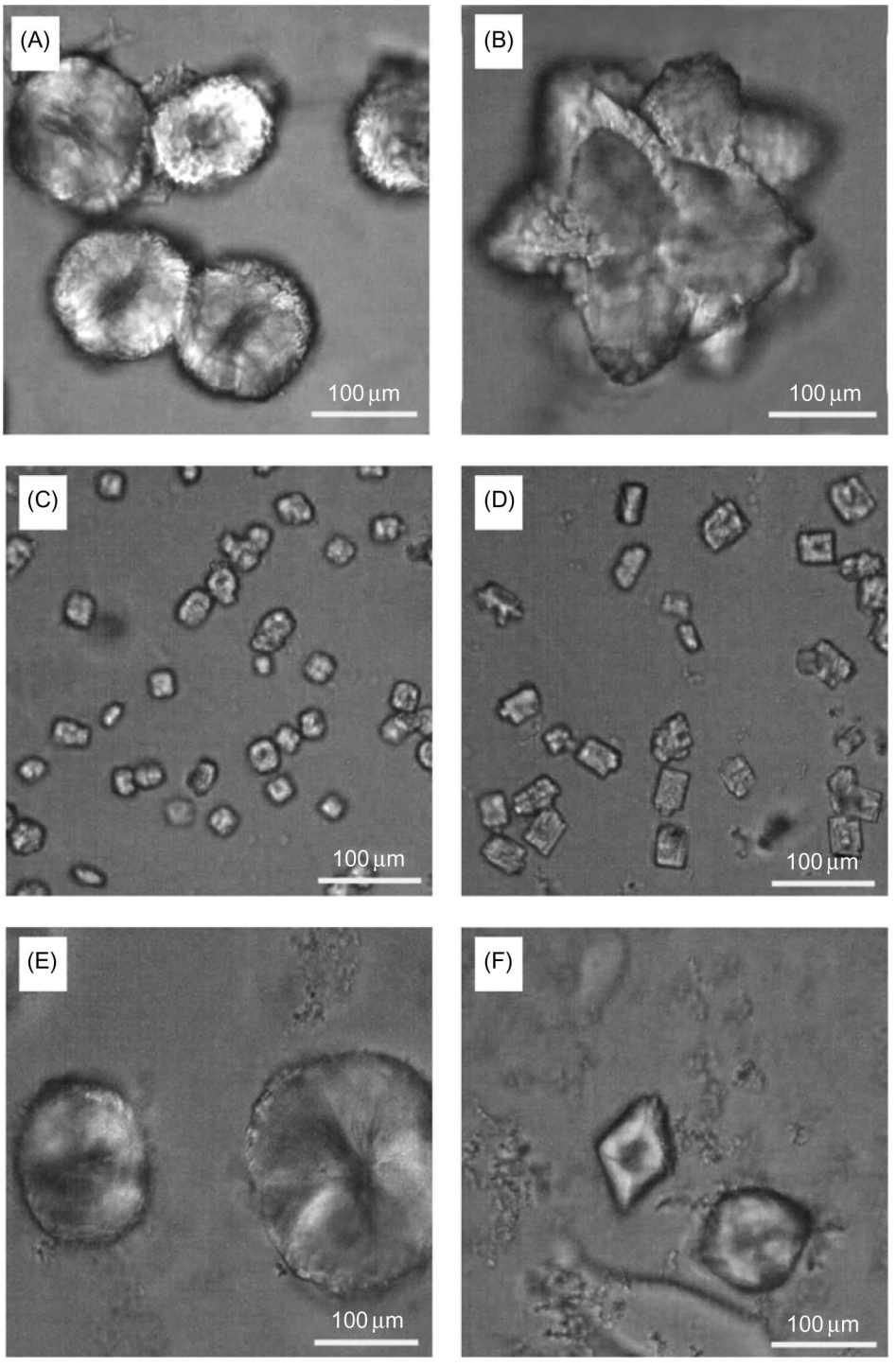

Figure 7. Different crystal morphologies were obtained with (A) PEG 2000, 50 mg/mL; (B) PEG 2000, 100 mg/mL; (C) PEG 4000, 50 mg/mL; (D) PEG 4000, 100 mg/mL; (E) PEG 6000, 50 mg/mL; and (F) PEG 6000, 100 mg/mL of PEG precipitants.

specific antiviral activity for the rhIFN before and after crystallization was 5.16×10^{13} and 5.25×10^{13} IU/mg, respectively. The percentage of cytopathic effect inhibition of crystallized rhIFN at optimal conditions was 101.8 ± 10.4%, suggesting that rhIFN has well kept its functional integrity during crystallization.

As well known when rhIFN α-2b is correctly folded, the conformational epitopes of the cytokine can be recognized by IgG1-subclass monoclonal antibodies (mAbs). The ELISA not only measures low concentrations (down to 0.2 ng/mL) of rhIFN α-2b but may also show rhIFN integrity and has been used in the process

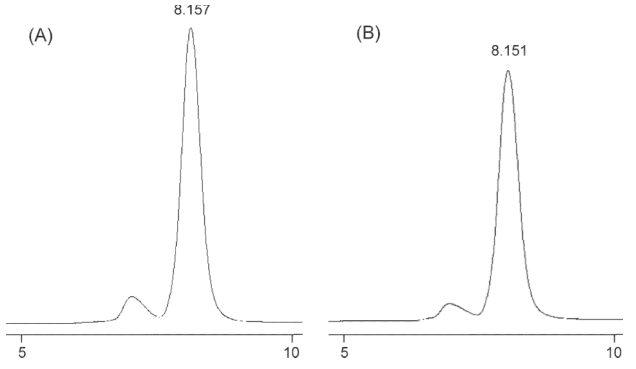

Figure 8. Chromatographs of rhIFN (A) before and (B) after crystallization by SEC-HPLC.

and quality control of large-scale production of rhIFN α-2b[21,22]. To know if our crystallization process influences the recognition of rhIFN α-2b by specific anti-rhIFN monoclonal antibodies, we assayed either native rhIFN α-2b or crystal samples by double-antibody sandwich-based ELISA test. The percentage of rhIFN after crystallization that has immunoactivity was 96.8 ± 9.9%, as detected by ELISA. It has been reported that there is a good dose–effect relationship between bioactivity and immunoactivity of cytokines[34]. Therefore, it could be concluded by both the antiviral activity and immunoactivity that the biological potency of rhIFN was not affected by the crystallization process.

In vitro release of rhIFN crystals

The in vitro release behaviors of rhIFN from crystals that produced at varied equilibration temperatures are shown in Figure 9. It was found that a significant biphasic release pattern characterized by an initial burst release and subsequent delayed release over 48 hours was observed in crystals obtained at 22°C. In this case, approximately 40% of the drug was immediately released at the first sampling time of 1.0 hour. As the crystal temperature decreased, the initial burst release was reduced significantly. Comparing with that of crystals obtained at 22°C, the cumulative release of the crystals produced at temperatures of 14°C and 4°C over the first sampling hour was 27.8% and 11.6%, respectively. Raising the temperatures of the crystallization systems would raise the solubility of rhIFN, which would lower the supersaturation degrees. The decreased supersaturation degrees provided relatively weaker driving forces in nucleation and crystal growth and might lead to production of crystal with looser structures which would be corroded more easily by outer ions. Therefore, as the equilibration temperatures rose from 4°C to 22°C, the produced crystals took on an increasingly higher dissolution rates.

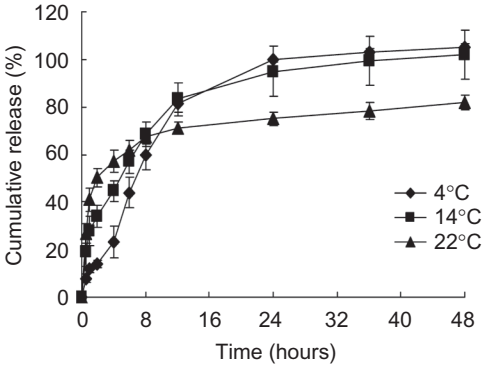

Figure 9. In vitro release profile of rhIFN from crystals produced at 4°C (◆), 14°C (■), and 22°C (▲) in PBS.

Conclusions

The morphology and quality of protein crystals are significantly affected by seeding concentration, pH of the hanging drop, ionic strength of the equilibration solution, and added precipitants. After the crystallization process, rhIFN maintained most of its biological potency. In vitro release from the crystals was found to depend on the crystal temperatures. The work described here suggested an exciting possibility of therapeutic protein crystals as a long-acting formulation.

Acknowledgments

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

References

1. Degim IT, Celebi N. (2007). Controlled delivery of peptides and proteins. Curr Pharm Des, 13:99–117.
2. Rathore N, Rajan RS. (2008). Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnol Prog, 24:504–14.
3. Greenwald RB, Choe YH, McGuire J, Conover CD. (2003). Effective drug delivery by PEGylated drug conjugates. Adv Drug Deliv Rev, 55:217–50.

4. Chemmanur AT, Wu GY. (2006). Drug evaluation: Albuferon-alpha–an antiviral interferon-alpha/albumin fusion protein. Curr Opin Investig Drugs, 7:750–8.
5. Sheshala R, Peh KK, Darwis Y. (2009). Preparation, characterization, and in vivo evaluation of insulin-loaded PLA-PEG microspheres for controlled parenteral drug delivery. Drug Dev Ind Pharm, 35:1364–74.
6. Sinha VR, Trehan A. (2003). Biodegradable microspheres for protein delivery. J Control Release, 90:261–80.
7. Basu SK, Govardhan CP, Jung CW, Margolin AL. (2004). Protein crystals for the delivery of biopharmaceuticals. Expert Opin Biol Ther, 4:301–17.
8. Jen A, Merkle HP. (2001). Diamonds in the rough: Protein crystals from a formulation perspective. Pharm Res, 18:1483–8.
9. Brader ML, Sukumar M, Pekar AH. (2002). Hybrid insulin cocrystals for controlled release delivery. Nat Biotechnol, 20:800–4.
10. Ferrantini M, Capone I, Belardelli F. (2007). Interferon-alpha and cancer: Mechanisms of action and new perspectives of clinical use. Biochimie, 89:884–93.
11. Samuel CE. (2001). Antiviral actions of interferons. Clin Microbiol Rev, 14:778–809.
12. Sánchez A, Tobío M, González L, Fabra A, Alonso MJ. (2003). Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha. Eur J Pharm Sci, 18:221–9.
13. Thote AJ, Chappell JT Jr, Gupta RB, Kumar R. (2005). Reduction in the initial-burst release by surface crosslinking of PLGA microparticles containing hydrophilic or hydrophobic drugs. Drug Dev Ind Pharm, 31:43–57.
14. Qiu J, Wei XH, Geng F, Liu R, Zhang JW, Xu YH. (2005). Multivesicular liposome formulations for the sustained delivery of interferon alpha-2b. Acta Pharmacol Sin, 26:1395–401.
15. Vyas SP, Rawat M, Rawat A, Mahor S, Gupta PN. (2006). Pegylated protein encapsulated multivesicular liposomes: A novel approach for sustained release of interferon α. Drug Dev Ind Pharm, 32:699–707.
16. Grace M, Youngster S, Gitlin G, Sydor W, Xie L, Westreich L, et al. (2001). Structural and biologic characterization of pegylated recombinant IFN–alpha2b. J Interferon Cytokine Res, 21:1103–15.
17. Glue P, Fang JW, Rouzier-Panis R, Raffanel C, Sabo R, Gupta SK, et al. (2000). Pegylated interferon-alpha2b: Pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Hepatitis C Intervention Therapy Group. Clin Pharmacol Ther, 68:556–67.
18. Foser S, Schacher A, Weyer KA, Brugger D, Dietel E, Marti S, et al. (2003). Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α-2a (PEGASYS). Protein Expr Purif, 30:78–87.
19. Luft JR, DeTitta GT. (1992). HANGMAN: A macromolecular hanging drop vapor diffusion technique. J Appl Crystallogr, 25:324–5.
20. Rubinstein S, Familletti PC, Pestka S. (1981). Convenient assay for interferons. J Virol, 37:755–8.
21. Santana H, Espino Y, Franco A, Furrazola G, Hardy E. (1999). A sandwich-type enzyme-linked immunosorbent assay for the analysis of recombinant human interferon α-2b. Biotechnol Tech, 5:341–6.
22. Chen X, Matteucci ME, Lo CY, Johnston KP, Williams RO 3rd. (2009). Flocculation of polymer stabilized nanocrystal suspensions to produce redispersible powders. Drug Dev Ind Pharm, 35:283–96.
23. Umeda Y, Fukami T, Furuishi T, Suzuki T, Tanjoh K, Tomono K. (2009). Characterization of multicomponent crystal formed between indomethacin and lidocaine. Drug Dev Ind Pharm, 35(7):843–51.
24. McPherson A. (1990). Current approaches to macromolecular crystallization. Eur J Biochem, 189:1–23.
25. Radhakrishnan R, Walter LJ, Hruza A, Reichert P, Trotta PP, Nagabhushan TL, et al. (1996). Zinc mediated dimer of human interferon-alpha 2b revealed by X-ray crystallography. Structure, 4:1453–63.
26. Wiencek JM. (1999). New strategies for protein crystal growth. Annu Rev Biomed Eng, 1:505–34.
27. Asherie N. (2004). Protein crystallization and phase diagrams. Methods, 34:266–72.
28. McPherson A. (1985). Crystallization of macromolecules: General principles. Methods Enzymol, 114:112–20.
29. Kantardjieff KA, Rupp B. (2004). Protein isoelectric point as a predictor for increased crystallization screening efficiency. Bioinformatics, 20:2162–8.
30. Santana H, Martínez E, Sánchez JC, Moya G, Sosa R, Hardy E. (1999). Molecular characterization of recombinant human interferon alpha-2b produced in Cuba. Biotecnol Apl, 16:154–9.
31. Smatanova IK. (2002). Crystallization of biological macromolecules. Mater Struct, 9:14–5.
32. Chayen NE. (1998). Comparative studies of protein crystallization by vapour-diffusion and microbatch techniques. Acta Crystallogr D Biol Crystallogr, 54:8–15.
33. Sauter C. (1999). Additives for the crystallization of proteins and nucleic acids. J Cryst Growth, 196:365–76.
34. Watanabe H, Hori A, Seno M, Kozai Y, Igarashi K, Ichimori Y, et al. (1991). A sensitive enzyme immunoassay for human basic fibroblast growth factor. Biochem Biophys Res Commun, 175:229–35.