Supporting Information

One-Month Zero-Order Sustained Release and Tumor Eradication after a Single Subcutaneous Injection of Interferon Alpha Fused with a Body-Temperature-Responsive Polypeptide

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Experimental Section

1. Materials. All molecular biology reagents were purchased from New England Biolabs unless otherwise specified. All chemical reagents were purchased from Sigma-Aldrich unless otherwise specified. All cell culture reagents and media were purchased from Gibco unless otherwise specified. Daudi B cells, human ovarian carcinoma OVCAR-3 cells and malignant melanoma C8161 cells were purchased from cell bank of Chinese Academy of Medical Sciences. Female BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China) and accommodated in animal research facility of Tsinghua University, and it is accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). All animal procedures applied in this research are approved by the Institutional Animal Care and Use Committee (IACUC).

2. Construction, expression and purification of IFN-ELP(V), IFN-ELP(A) and IFN. The gene encoding IFN (NCBI GI 386795 for protein) was PCR-amplified from a previously synthesized IFN-containing pGEM®-T vector (Sangon Biotech, China) and inserted into pET-25b (+) (Novagen) using *Nde* I/*Eco* RI restriction sites. The genes encoding IFN-ELP(V) and IFN-ELP(A) were constructed and inserted into pET-25b (+) vector by using the PRe-RDL method. The sequences of ELP(V) and ELP(A) were composed of 90 repeats of the pentapeptide Val-Pro-Gly-Val-Gly and Val-Pro-Gly-Ala-Gly, respectively. After verified by DNA sequencing, the constructed plasmids encoding IFN-ELP(V), IFN-ELP(A) and IFN were transformed into *E. coli* strain Rosetta-gami (DE3) pLysS competents (Invitrogen) and incubated in Luria Bertani medium containing 50 μg/mL ampicillin at 37 ˚C. The cultures were used to inoculate 1 L of sterile terrific

broth medium with 250 rpm shaking until the optical density at 600 nm (OD_{600}) was 0.5, at which time the temperature was lowered to 25 ˚C and isopropyl-β-D-thiogalactopyranoside (IPTG) with a final concentration of 500 μM was added for expression induction overnight. Cells were collected and suspended in PBS solution, pH = 7.4 and lysed by sonication. The cell lysates were centrifuged. Nucleic acids in the extracts were precipitated by adding polyethyleneimine (1% w/v). After centrifugation, the pellets were discarded.

IFN-ELP(V) and IFN-ELP(A) in the supernatants were purified by inverse transition cycling (ITC). The inverse phase transitions were initiated by the addition of NaCl to a final concentration of 3 M and the aggregated IFN-ELP(V) and IFN-ELP(A) were separated from the solutions by centrifugation at 16,000 × g at 37 ˚C for 15 min. The pellets were resuspended in cold PBS on ice for 15 min. The suspensions were centrifuged at 4 ˚C to remove any insoluble particles. This cycle was typically repeated 2 times. Purified IFN-ELP(V) and IFN-ELP(A) were stored in PBS, pH = 7.4 at -80 ˚C for further use.

IFN was purified by immobilized metal affinity chromatography. In brief, the supernatant was applied to a 5 mL HisTrap column (GE Healthcare) mounted in AKTA purifier system. The column was washed with equilibration buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 5 mM imidazole, pH 7.4) and then washing buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 50 mM imidazole, pH 7.4). His_6-tagged IFN protein was finally eluted using the buffer containing 500 mM imidazole. The eluted IFN was further purified on a HiPrep 26/10 desalting column (GE Healthcare) for buffer exchanging to 10 mM PBS, pH 7.4 and stored at -80 ˚C for further use.

The purification process was monitored with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the bicinchoninic acid (BCA) assay was used to determine the protein concentrations.

3. **Cell culture.** OVCAR-3, C8161 and Daudi B cells were cultured in RPMI-1640 medium containing 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hyclone) at 37 ˚C in a humidified, 5% CO_2 atmosphere.

4. *In vitro* antiviral activity.

4.1. Cell cultures and IFN-α treatments. The HepG2-derived HBV-producing stable cell line HepAD38 was maintained in Dulbecco's modified eagle medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1μg/mL doxycycline (Dox). HepAD38 cells were seeded in a 24-well plate at 1×10^5 cells per well and Dox was withdrawn from the culture medium 4 d before cell plating to initiate HBV replication and virus particle secretion. Commercial recombinant human IFN-α2b (Novoprotein, Shanghai, China) was added directly to the culture medium every 48 h at a final concentration of 1000 IU/mL. Similarly, IFN, IFN-ELP(A) and IFN-ELP(V) were added at a final concentration of 1000 ng/mL.

4.2. Quantification of HBeAg and HBV DNA. The cell culture supernatant was harvested after 6 d of IFN treatment, and the level of HBeAg was detected by a time-resolved fluoroimmunoassay (TRFIA) according to manufacturer's instructions (PerkinElmer, Shanghai, China).[1] The levels of HBV DNA in culture medium were determined by quantitative PCR (qPCR) in Applied Biosystems StepOne plus Real-Time

PCR Systems (Applied Biosystems, Mannheim,USA). Amplifications were performed as follows: 94 ˚C for 10 min, followed by 40 cycles at 94 ˚C for 15 s and 58 ˚C for 40 s.

5. Biodistribution. Female BALB/c nude mice of 6 weeks old were subcutaneously inoculated in the right dorsal area with 5×10^6 OVCAR-3 cells (0.2 mL) suspended in RPMI-1640 medium. When the tumors grew to a size of 100-150 mm^3 (~ six weeks), the mice were randomly grouped into 3 groups (3 mice in each group) and received subcutaneous injections of IFN, IFN-ELP(A) and IFN-ELP(V) at their MTDs, respectively. At 24 h, 72 h and 30 d after the injections, the mice were sacrificed by CO_2 and major tissues (tumor, heart, kidney, liver, spleen, lung, pancreas, stomach, muscles and intestine) were harvested. The collected tissues were then weighed, homogenized and suspended in the corresponding quantity of 10 mM PBS extraction buffer containing 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, phosphatase inhibitor cocktail 2 and 3 (1:100 diluted) and protease inhibitor cocktail (1:100 diluted). The concentrations of IFN in samples were quantified by ELISA as described above. The background of tissues from untreated mice was subtracted from the acquired data correspondingly. The data were presented as IFN equivalent (ng) per gram of tissue (ng/g tissue).

6. Biological safety. To evaluate the *in vivo* safety of IFN, IFN-ELP(A) and IFN-ELP(V), ovarian tumor-bearing mice were sacrificed on the 9th day post subcutaneous injections at their MTDs, tissues including tumor, heart, kidney, spleen, lung and liver were collected. The harvested tissues were fixed with formalin, and embedded in paraffin for histological examination. Tissue sections (5 μm in thickness) mounted onto glass slides were stained with hematoxylin-eosin (H&E) for morphology observation according to

standard procedures. The images of all sections were captured with a Nikon Eclipse 90i microscope.

At the end of the treatments, blood was collected via retro orbital for hematology examination. Hematological parameters of complete blood were measured with a Hematology Analyzer (SYSMEX), including number counts of white blood cells (WBC), red blood cells (RBC), platelets (PLT) and the concentration of hemoglobin (HGB). Clinical biochemistry parameters of serum were measured by Automatic Biochemical Analyzer (HITACHI), including lactate dehydrogenase (LDH) and creatine kinase isoenzymes (CK-MB) which are markers for heart function, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) which are markers for hepatic function, creatinine (CREA) and blood urea nitrogen (UREA) which are markers for kidney function.

Blood (1 mL) was collected in heparinized-tubes via retro orbital from female healthy BALB/c nude mice. The supernatant was discarded after centrifuging for 10 min at 3,000 g and red blood cells (RBC) were washed with PBS. RBCs were then suspended in 10 mL PBS. 500 μL of RBCs supernatant was added into 500 μL PBS buffer containing different concentrations of IFN, IFN-ELP(A) and IFN-ELP(V) (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL IFN-equivalent). The hemolysis ratios of 500 μL RBCs supernatant mixed with 500 μL PBS and distilled water were valued as 0% and 100%, respectively. After incubating for 2 h at 37℃, the mixture was centrifuged as mentioned above and the obtained supernatant was measured at the absorbance of 540 nm using a SpectraMax M3 Microplate Reader. The formula was used to calculate the hemolysis ratio: hemolysis% = [(sample absorbance − negative control) / (positive control − negative control)] × 100%.

Supplementary Figures and Tables

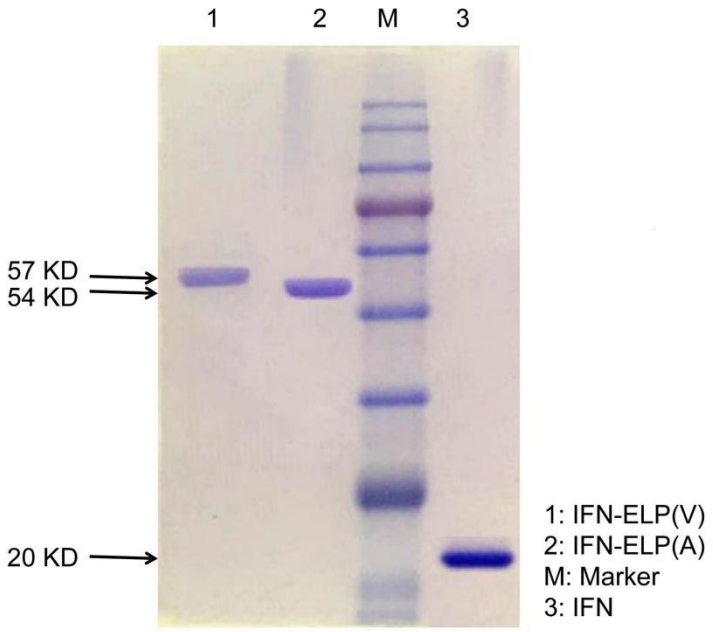

Figure S1. SDS-PAGE analyses of IFN-ELP(V), IFN-ELP(A) and IFN after purification by ITC.

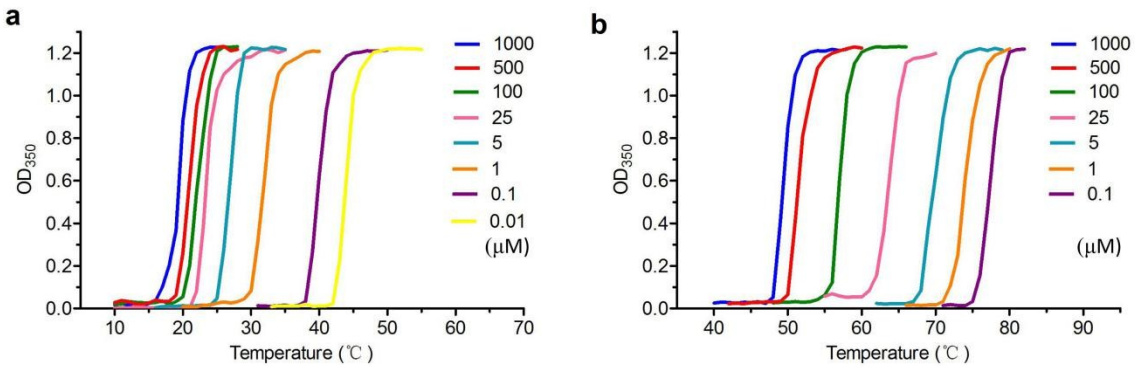

Figure S2. Turbidity profiles of IFN-ELP(V) a) and IFN-ELP(A) b) as a function of temperature at different concentrations.

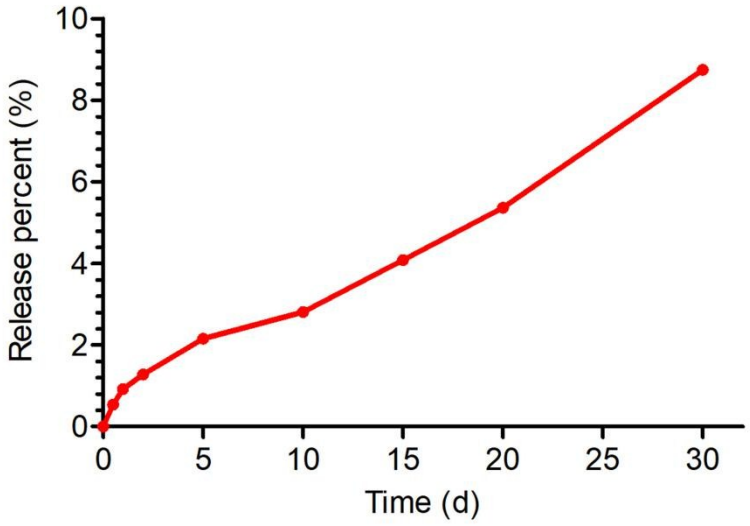

Figure S3. *In vitro* release profile of IFN-ELP(V) at 37℃ for a month.

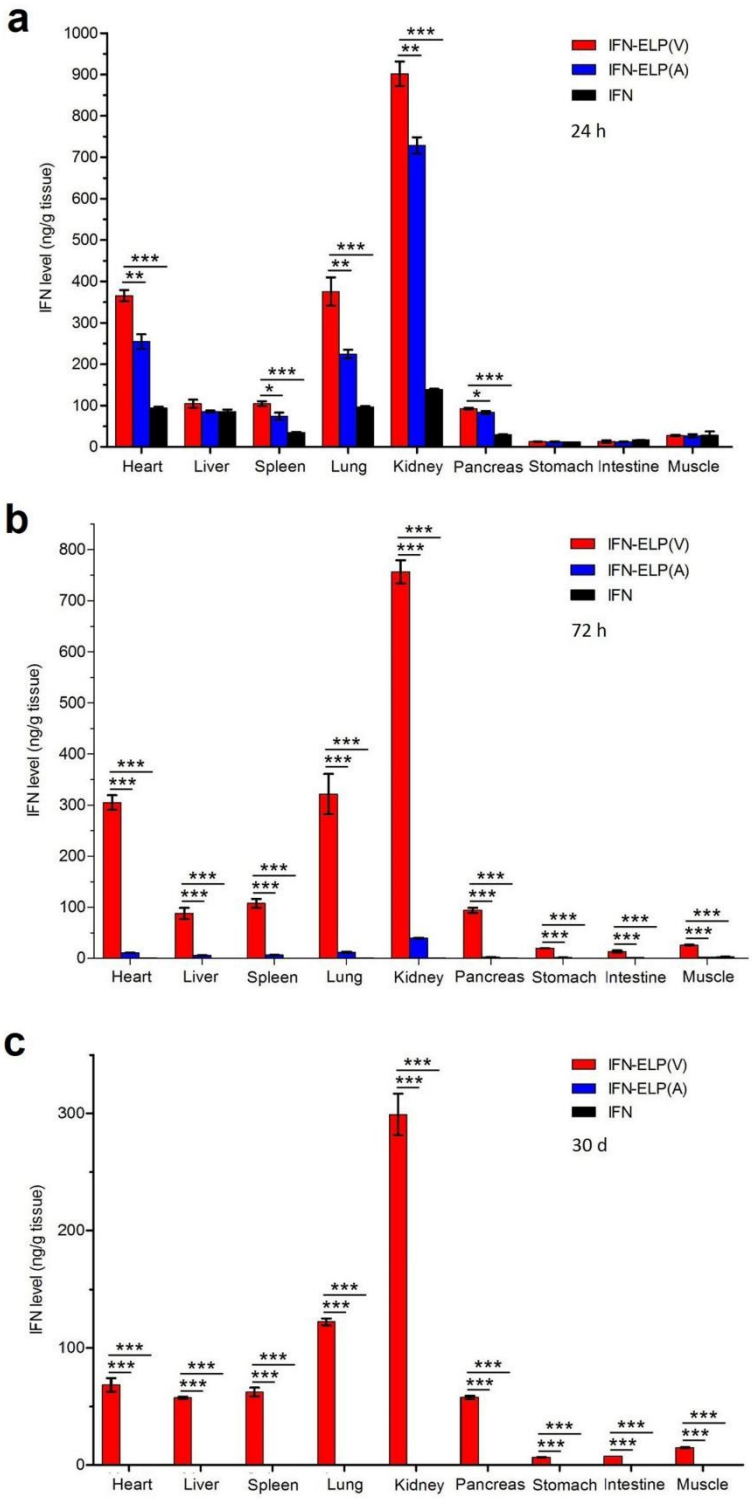

Figure S4. *In vivo* biodistribution of IFN-ELP(V), IFN-ELP(A) and IFN at 24 h (a), 72 h (b) and 30 d (c) post administration (n = 3,**P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant difference for IFN-ELP(V) compared with IFN-ELP(A) and IFN). Data are shown as mean ± standard error of the mean.

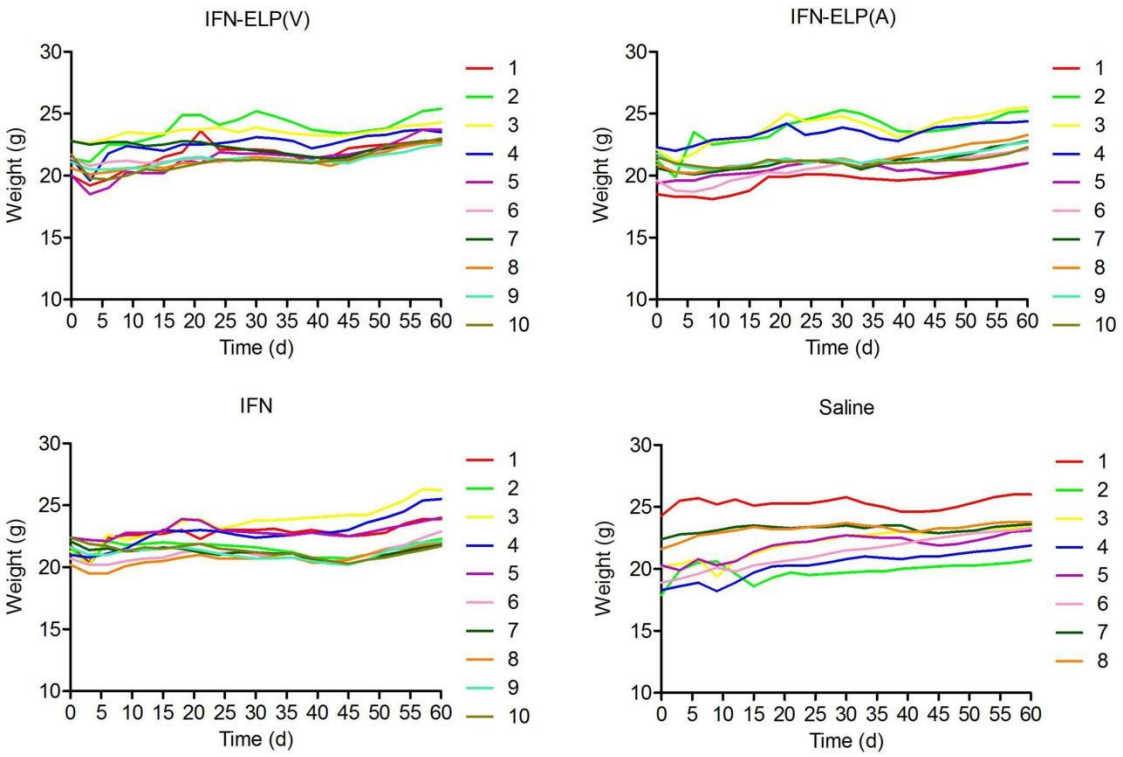

Figure S5. The change of mouse body weight post administration.

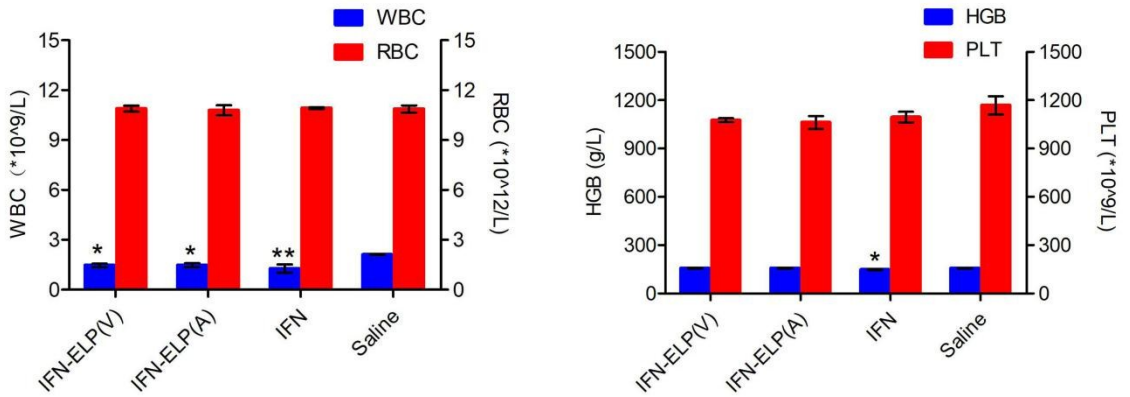

Figure S6. Hematological examination for mice post administration. WBC, white blood cells; RBC, red blood cells; PLT, platelets; HGB, hemoglobin (n = 3, **P* < 0.05, ***P* < 0.01).

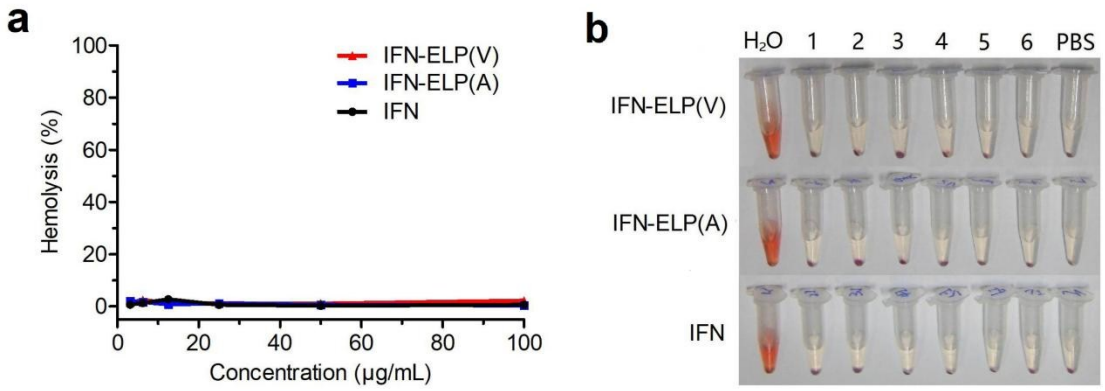

Figure S7. Hemolysis analysis. a) Hemolytic effect of IFN-ELP(V), IFN-ELP(A) and IFN on red blood cells (n = 3). Data are shown as mean ± standard error of the mean. b) Digital images of the hemolytic effect. The numbers 1 to 6 represent concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 μg/mL IFN-equivalent.

Table S1. Pharmacokinetic parameters of IFN-ELP(V), IFN-ELP(A) and IFN after intravenous injections at the same dose of 30 μg IFN-equivalent/kg BW (0.1 μM) (n = 3).

Parameter	IFN	IFN-ELP(A)	IFN-ELP(V)
Terminal half-life $T_{1/2\beta}$ (h)	1.2 ± 0.08	9.8 ± 0.4	9.9 ± 0.24
Area under curve AUC (mg/L·h)	12.4 ± 1.01	147.1 ± 7.5	150.4 ± 8.7
MRT (h)	0.8 ± 0.2	11.7 ± 0.73	12.6 ± 1.2
Tmax (h)	0.017 ± 0	0.017 ± 0	0.017 ± 0
Cmax (μg/L)	24.3 ± 0.009	26.3± 0.09	26.3 ± 0.09
Elimination rate constant K10 (1/h)	2.2 ± 0.2	0.18 ± 0.01	0.18± 0.01
Clearance CL (mL/h)	48.7 ± 4.0	4.0 ± 0	4.0 ± 0

Note: The data are presented as IFN-equivalent.

Table S2. Pharmacokinetic parameters of IFN-ELP(V), IFN-ELP(A) and IFN after subcutaneous injection at their MTDs (n = 3).

Parameter	IFN	IFN-ELP(A)	IFN-ELP(V)
Half-life time $T_{1/2}$ (h)	1.9 ± 0.13	10.5 ± 1.5	497.2± 57.0 (20.7 d)
Area under curve AUC (mg/L·h)	45.1 ± 7.1	135.4 ± 10.2	3176 ± 247
MRT (h)	5.1 ± 0.21	18.0 ± 0.67	410.0 ± 25.3
Tmax (h)	2.7 ± 1.2	4 ± 0	13.3 ± 9.2
Cmax (μg/L)	5457 ± 759	6131± 262	5461 ± 559
Elimination rate constant Ke (1/h)	0.37 ± 0.03	0.067 ± 0.009	0.0013 ± 0.0006
Clearance CL (mL/h)	7.0 ± 1.0	3.7 ± 0.58	1.0 ± 0

Note: The data are presented as IFN-equivalent.

Reference

1 H. Huang, J. Wang, W. Li, R. Chen, X. Chen, F. Zhang, D. Xu, F. Lu, *J. Clin. Virol.*, 2018, **99-100**, 71-78.