**Supporting information**

**for**

# Therapeutic Protein PEPylation: the Helix of Nonfouling Synthetic Polypeptides Minimizes the Anti-Drug Antibody Generation

Yingqin Hou[a], Yu Zhou[a], Hao Wang[a], Jialing Sun[a], Ruijue Wang[a], Kai Sheng[a], Jingsong Yuan[a], Yali Hu[b], Yu Chao[c], Zhuang Liu[c] and Hua Lu[a]*

Email: chemhualu@pku.edu.cn

[a]Beijing National Laboratory for Molecular Sciences, Center for Soft Matter Science and Engineering, Key Laboratory of Polymer Chemistry and Physics of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, People's Republic of China

[b]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, People's Republic of China

[c] Institute of Functional Nano & Soft Materials (FUNSOM), Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou, Jiangsu 215123, China

**Materials.** All chemicals were purchased from commercial sources and used as received unless otherwise specified. No unexpected or unusually high safety hazards were encountered. Phenyl trimethylsilyl sulfide (PhS-TMS) was purchased from Sigma-Aldrich (St. Louis, USA). Anhydrous *N,N*-dimethylformamide (DMF) was purchased from Sigma-Aldrich and treated with methyl isocyanate bounded polystyrene beads (Sigma-Aldrich, St. Louis, USA) prior to polymerization. Methoxypoly(ethylene glycol) carboxyl acid (mPEG-COOH, molecular weight: ~20000) was purchased from JenKem Technology Co. Ltd (Beijing). γ-(2-(2-(2-methoxyethoxy)ethoxy)ethyl L-glutamate NCA (EG$_3$GluNCA), wild-type and mutant IFN were produced according to established protocols.[1,2] The plasmid of wild-type human growth hormone (GH) was obtained as a generous gift from Prof. Demin Zhou (Peking University). The absolute molecular weight (MW) of L-P(EG$_3$Glu) and DL-P(EG$_3$Glu) was calculated based on the *d*n/*d*c values previously reported.[2] For *in vivo* studies, endotoxin affinity beads were used to remove endotoxin from all protein variants. The final endotoxin concentration was measured by Toxin Sensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript, Nanjing, China). The results showed that the endotoxin level was less than 0.1 EU/mL, significantly below the safety limit required for medical devices and parenteral drugs (0.5 EU/mL according to FDA of U.S.A.).

**Instrumentation.** Tandem gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA), a DAWN HELEOS 9-angle laser light scattering detector (Wyatt Technology, Santa Barbara, CA) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). The detection wavelength of HELEOS was set to 658 nm. Separations were performed on serially connected size exclusion columns (10$^3$Å, 10$^4$Å, 10$^5$Å Phenogel columns, 5 μm, 7.8 × 300 mm, Phenomenex, Torrance, CA) at 50 °C using 0.1 M LiBr in DMF as the mobile phase. FPLC separation was performed on an ÄKTA FPLC system (GE Healthcare, Inc.) using a Superdex 200 Increase 10/300 GL column or MonoS 5/50 GL column. Protein concentration was quantified by NanoPhotometer™P-class (Germany). SDS-PAGE gel was recorded on a typhoon FLA 9500 laser scanner (GE Healthcare Corp.). Circular dichroism spectroscopy was recorded on a CD spectrometer (Bio-Logic Science Instruments, France; wavelength: 200-250 nm; scan rate: 100 nm/min; band width: 1 nm; scan point interval: 1.0 nm; Dynode voltage values: 200~400 V,

temperature: 37 °C, concentration: 0.15 mg/mL based on protein). Thermofluor assays of the protein and conjugates were measured by a Light Cycler 96 Real-time PCR System (Roche, Switzerland) and the melting temperature ($T_m$) was calculated by the built-in software. Cytotoxicity studies were performed by using an EnSpire® Multimode Plate Reader (PerkinElmer, USA). Flow cytometry study was performed on a BD LSR Fortessa flow cytometer equipped with 405, 488 and 640 nm lasers (BD Bioscience. U.S.A.). Surface plasmon resonance (SPR) analysis was performed on a Biacore T200 (GE Healthcare).

**Cell line and animals**. Human B lymphoma Daudi cells were cultured in 1640 medium (Corning, Manassas, USA) supplemented with 10% FBS, 100 U mL$^{-1}$ of penicillin and 100 U mL$^{-1}$ of streptomycin. Human ovarian carcinoma OVCAR-3 was grown in 1640 medium supplemented with 20% FBS, 100 U mL$^{-1}$ of penicillin, 100 U mL$^{-1}$ of streptomycin and 0.01 mg/mL bovine insulin. The patient prostate tumor tissue was acquired from a patient in SuZhou Municipal Hospital after obtaining informed consent and used under the Good Clinical Practice (GCP) approved by China Food and Drug Administration (CFDA). BALB/c nude mice, female C57 mice and female Sprague-Dawley rats were purchased from Vital River Laboratories (Beijing, China). All animal experiments were performed in compliance with the Guideline for the Care and Use of Laboratory Animals, and were approved by the Experimental Animal Ethics Committee in Beijing.

**Synthesis of L- and DL-P(EG$_3$Glu).** In a glovebox, L-EG$_3$-GluNCA (82.2 mg, 0.258 mmol, 100 equiv) in anhydrous DMF (800 μL) was mixed with phenyl trimethylsilyl sulfide (PhS-TMS, 5.1 μL, 0.5 M, 1.0 equiv). After stirring at room temperature for 48 h, the desired product denoted as L-P(EG$_3$Glu) was recovered by precipitation in diethyl ether (40 mL), washed with diethyl ether (40 mL × 2), and passed through a PD-10 SEC column. The final product was obtained as a colorless gum-like solid after lyophilization (52 mg, yield 71%). DL-P(EG$_3$Glu) were synthesized by following a similar protocol except that racemic DL-EG$_3$-GluNCA were used as the monomer. Both polymers were characterized by GPC and $^1$H NMR.

**Synthesis of mPEGCOS$^n$Bu.** mPEG$_{20k}$-COOH (200 mg, 1.0 equiv), $n$-butyl mercaptan (32 μL, 30.0 equiv) and $N,N'$-dicyclohexylcarbodiimide ( 14.4 mg, 7.0 equiv) were dissolved in dry dichloromethane (2.0 mL) and stirred at room temperature for 24 h. The crude product was precipitated in diethyl ether (40 mL) and washed with diethyl ether (40 mL × 2). The final product was purified with a PD-10 SEC column and obtained as a white solid (~90% yield) after lyophilization.

**Synthesis of the protein-polymer conjugates.** The synthesis of L$_{20k}$-IFN was used here as an example. Typically, Cys-IFN (5.0 mg, 1.0 equiv) was mixed with L-P(EG$_3$Glu) (3.0 equiv) in Tris·HCl buffer (50 mM, 500 μL, pH 7.4) and incubated at room temperature for 10~12 h. The product was purified via FPLC on a Superdex 200 Increase size exclusion column. The residual free polymer in the product was removed by a NiNTA column. The overall purified yield was ~67% based on IFN. DL$_{20k}$-IFN were synthesized by following a similar protocol, yield ~52%.

PEG$_{20k}$-IFN was synthesized following a similar protocol with mPEGCOS$^n$Bu (5.0 equiv) and purified with a monoS 5/50 GL column, yield ~49%.

The GH series L$_{20k}$-GH, DL$_{20k}$-GH and PEG$_{20k}$-GH were also synthesized by following the same protocol, purification yield ~50-70%.

**Thermofluor assay**. Thermofluor assay of wt-IFN and the IFN-polymer conjugates was based on measuring the fluorescence in yellow555 panel (excitation = 533 nm, emission = 572 nm) on a Roche LightCycler96. Briefly, 5 μL 200 × Sypro orange protein gel stain (Thermo) and 45 μL protein (5~10 μM in 1 ×PBS buffer) were mixed together and added to a 96-well plate in triplicate. The samples were heated from 37 to 98 °C at a rate of 2.2 °C/min. $T_m$ was calculated by the built-in software.

**Trypsin resistance assay.** FAM labeled $L_{20k}$-IFN, $DL_{20k}$-IFN or $PEG_{20k}$-IFN (final concentration: 0.22 mg/mL of IFN) in Tris-HCl buffer (50 mM, pH 7.4) was incubated with trypsin (0.01 mg/mL, 0.01 equiv) at 37 °C. At each predetermined time point, an aliquot was sampled and the reaction was terminated by boiling at 95 °C for 10 min. The samples were then analyzed on the same SDS-PAGE gel. Degradation of the conjugates was evaluated by calculating the relative fluorescent intensity of each band by using a typhoon FLA laser scanner. For the evaluation of wt-IFN degradation, a similar procedure as above was used, with the exception that protein quantitation was based on Coomassie blue staining.

The trypsin resistance assay of wt-, $L_{20k}$-, $DL_{20k}$- and $PEG_{20k}$-GH was performed following the similar protocol.

**Surface plasmon resonance (SPR) analysis.** The binding affinity of wt-, $L_{20k}$-, $DL_{20k}$- and $PEG_{20k}$-IFN to IFNAR2 (Sino Biological) was determined on a BIAcore T200 (GE Healthcare) with a CM5 chip. The receptor IFNAR2 was immobilized on the chip using a standard amine coupling method (Biacore; GE Healthcare BioSciences) and approximately 500–800 RUs of soluble IFNAR2 was immobilized. Various concentrations of IFN variants (0.1 – 40 nM) in HBS-EP buffer (Biacore; 10 mM HEPES, 3 mM EDTA, 150 mM NaCl, pH 7.4, 0.005% Surfactant P20) were injected over the IFNAR2 surface at a flow rate of 30 μL/min for 120 s. Dissociation was monitored for 360 s post injection. The subsequent regeneration was performed using Gly-HCl (10 mM, pH3.0) for 15 s.

To determine the individual rate constant, the sensograms were fit to the BIA evaluation software (T200 version1.0) using a simple 1:1 binding kinetic model.

**Daudi cell proliferation inhibition assay.** Daudi cells were seeded at a density of 5000 per well in a 96-well plate and incubated with gradient concentrations of IFN variants for 72 h ($n = 3$). The relative cell viability of each group was determined by Cell Titer-Blue® Viability Assay (Promega, US) following the manufacturer's protocol. Data were fitted using the GraphPad Prism 5.0 software and $IC_{50}$ values were expressed as mean ± S.D.

**Pharmacokinetics**. Female Sprague-Dawley rats with an average body weight of ~250 g were used

in the pharmacokinetic study. The rats were randomly divided into four groups ($n$ = 3 or 6) and injected with wt-, $L_{20k}$-, $DL_{20k}$- or $PEG_{20k}$-IFN (0.2 mg/kg based on IFN) through a jugular cannulated vein. At selected time points (1, 15, 30 min, 1, 3, 6, 9, 12, 24, 48 and 72 h), blood (100 µL each time) was withdrawn from the cannulated jugular vein and allowed to stand at 4 °C for 30 min, followed by centrifugation at 4000 $g$ for 15 min. Plasma was separated and stored at -80 °C before analysis. The concentration of IFN was determined by using a human IFN ELISA Kit (eBioscience). Pharmacokinetic parameters were analyzed by DAS 3.0 software and expressed as mean ± S.D.

***In vivo* anti-tumor efficacy**. For the OVCAR-3 ovarian tumor model, human OVCAR-3 cells were cultured and implanted into female BALB/c nude mice as described above. The tumors were allowed to grow to ~40 mm$^3$ (~ 3-4 weeks) and the mice were randomly grouped (n = 5~7 per group, day 0). The tumor-bearing mice were intravenously (i.v.) administered with PBS saline, wt-IFN, $L_{20k}$-IFN, and $DL_{20k}$-IFN at a dose of 0.5 mg/kg for 5 times in total.

For the patient derived xenografted tumor model (PDX), the fresh patient's prostate tumor tissues after surgical resection were remained in growth medium and cut into pieces (~2-4 mm) in a sterile dish. The tumor pieces were implanted onto the back of male BALB/c nude mouse. The treatment was started when the tumor grew up to ~50 mm$^3$. The mice were intravenously (i.v.) administered with PBS saline, wt-IFN, $L_{20k}$-IFN, $DL_{20k}$-IFN and $PEG_{20k}$-IFN at a dose of 0.75 mg/kg every five days.

Tumor volume was calculated by the following formula:

$$V = L*W^2/2$$

Statistical analyses were performed using GraphPad Prism software 5.0 and data were expressed as mean ± S.D.

**Immunization and ELISA analysis for antibody titers**. Sprague-Dawley rats (~250g) were subcutaneously administered with $L_{20k}$-, $DL_{20k}$- or $PEG_{20k}$-IFN (0.2 mg/kg based on IFN, once per week for 4 weeks; the dosage interval was selected based on the half-life of each drug). Sera were collected weekly staring from week 0.

**For the time dependent anti-IFN IgG and IgM analysis:** 96-well plates were coated with wt-IFN (100 ng/well) at 4 °C overnight. Before the experiment, the plates were washed with the Wash Buffer A (200 μL/well) three times and blocked with the Assay Buffer A (200 μL/well) for 1 h at room temperature. Sera collected before (week 0) and after (week 1-4) the immunization were diluted with 1×PBS (100 μL/well, pH7.4) and added to the plates at a $10^4$-fold dilution for IgG analysis or a 500-fold dilution for IgM analysis. The plates were incubated at room temperature for 1 h. After the same washing and blocking procedures as described previously, the plates were incubated with a goat anti-mouse IgG-HRP (1/2000 dilution) or a goat anti-mouse IgM-HRP (1/2000 dilution) in the Assay Buffer (100μL/well) for another 1 h at room temperature. Finally, the plates were washed and then treated with TMB ELISA substrate (CWBIO) at room temperature for 5 min. The reaction was stopped by adding 2 N $H_2SO_4$ (100 μL/well) and the anti-IFN antibody levels were measured by reading the absorbance at 450 nm on an EnSpire® Multimode Plate Reader.

**For the analysis of anti-polymer antibodies:** A cross-coating ELISA was performed for the detection of anti-polymer antibody analysis. Briefly, $L_{20k}$-GH in 1×PBS (100 ng based on GH/well) was coated on the plate at 4 °C overnight for the measurement of anti-L-P($EG_3$Glu) antibodies in the sera immunized with $L_{20k}$-IFN, or vice versa. Similar cross coatings were performed for the analysis of anti-DL-P($EG_3$Glu) and anti-PEG antibodies. Before the experiment, the plates were

washed with the Wash Buffer B (200 μL/well) three times and blocked by the Assay Buffer B (200 μL/well) at room temperature for 1 h. Sera collected before (week 0) and after (week 1-4) the immunization were diluted 200 fold with Assay Buffer B, added to the plates (100 μL/well), and incubated at room temperature for 1.0 h. After the same washing and blocking procedures as described above, the plates were incubated with a goat anti-mouse IgG-HRP (1/2000 dilution) or a goat anti-mouse IgM-HRP (1/2000 dilution) in the Assay Buffer B (100 μL/well) for another 1.0 h at room temperature. Finally, the plates were washed, treated with TMB ELISA substrate (CWBIO) and incubated at room temperature for 5~10 min. The reaction was stopped by adding 2 N $H_2SO_4$ (100 μL/well), and the levels of anti-polymer antibodies were measured by reading the absorbance at 450 nm on an EnSpire® Multimode Plate Reader.

**DL-P(EG₃Glu) and PEG competition ELISA:** taking the free PEG competition as an example, $PEG_{20k}$-GH in 1×PBS was incubated separately in 96-well plates (100 ng based on GH/well) at 4 °C overnight. Before the experiment, the plates were washed with the Wash Buffer B (200 μL/well) three times and blocked by the Assay Buffer B (200 μL/well) at room temperature for 1 h. Sera collected after the 4th immunization of $PEG_{20k}$-IFN were diluted 200 fold with the Assay Buffer B and mixed with $mPEG_{20K}$ at gradient concentrations before adding to the plates (100 μL/well) and incubated at room temperature for 1.0 h. After the same washing and blocking procedures as described above, the plates were incubated with a goat anti-mouse IgG-HRP (1/2000 dilution) or a goat anti-mouse IgM-HRP (1/2000 dilution) in the Assay Buffer B (100 μL/well) for another 1.0 h at room temperature. Finally, the plates were washed, treated with TMB ELISA substrate (CWBIO) and incubated at room temperature for 5~10 min. The reaction was stopped by adding 2 N $H_2SO_4$

(100 µL/well), and the levels of antibodies were measured by reading the absorbance at 450 nm on an EnSpire® Multimode Plate Reader. DL-P(EG₃Glu) competition ELISA was performed in a similar way.

**Series dilution for the determination of anti-IFN antibody titers** (Figure S9): 96-well plates were coated with wt-IFN (100 ng/well) at 4 °C overnight. Before the experiment, the plates were washed with the Wash Buffer A (200 µL/well) three times and blocked with the Assay Buffer A (200 µL/well) for 1 h at room temperature. The sera collected in week 4 (one week after the 4th immunization) were diluted at series concentrations in 1×PBS (pH7.4) and incubated with the plates at room temperature for 1.0 h. The plates were washed with the Wash Buffer A (200 µL/well) three times, and then incubated with a goat anti-mouse IgG-HRP (CWBIO, 1/2000 dilution) or a goat anti-mouse IgM-HRP (1/2000 dilution) in the Assay Buffer A (100 µL/well) for 1 h at room temperature. The plates were subsequently washed with the Wash Buffer A (200 µL/well) for three times and treated with TMB ELISA substrate (CWBIO). The reaction was stopped by adding 2 N $H_2SO_4$ (100 µL/well) after incubation at room temperature for 5~10 min and the antibody titers were measured by reading the absorbance at 450 nm on an EnSpire® Multimode Plate Reader (PerkinElmer, USA).

The immunization of $L_{20k}$-GH, $DL_{20k}$-GH, or $PEG_{20k}$-GH was performed by following a similar protocol with a dose of 0.4 mg/kg. Apart from the regular blood collection every week, sera were also collected at selected time points after the 1st and 3rd infusion for the study of accelerated blood clearance effect. The plasma GH level was analyzed by Human Growth Hormone ELISA kit

(Boster). The ELISA analyses of anti-GH and anti-polymer antibodies in sera extracted from rats were performed in a similar protocol as described above.

Recipes of buffers used in ELISA:

$1 \times$PBS: 1.76 mM $KH_2PO_4$, 8.24 mM $Na_2HPO_4$, 2.68 mM KCl, 137 mM NaCl, pH7.4.

*Wash Buffer A*: 1.76 mM $KH_2PO_4$, 8.24 mM $Na_2HPO_4$, 2.68 mM KCl, 137 mM NaCl, pH7.4, 0.1% v/v Tween 20.

*Assay Buffer A*: 1.76 mM $KH_2PO_4$, 8.24 mM $Na_2HPO_4$, 2.68 mM KCl, 137 mM NaCl, pH7.4, 0.1% Tween 20, 0.5% BSA.

*Wash Buffer B*: 1.76 mM $KH_2PO_4$, 8.24 mM $Na_2HPO_4$, 2.68 mM KCl, 137 mM NaCl, pH7.4.

*Assay Buffer B*: 1.76 mM $KH_2PO_4$, 8.24 mM $Na_2HPO_4$, 2.68 mM KCl, 137 mM NaCl, pH7.4, 0.1% Tween 20, 0.5% BSA.

**Warning: TWEEN was excluded from all buffers in the anti-polymer antibody ELISA assays.**

**BMDC uptake and activation.** BMDCs were prepared by following a reported protocol.[3] Briefly, $1.0 \times 10^6$ bone marrow cells extracted from the femurs and tibiae of 4-5 weeks old male C57BL/6 mice were cultured in RPMI 1640 supplemented with 10% FBS, IL-4 (20 ng/mL), and GM-SCF (10 ng/mL). The medium was replaced every two days and the cells were used after a six-day in vitro culture. For the internalization experiment, BMDCs were incubated with FAM-labeled wt-, $L_{20k}$-GH, $DL_{20k}$-GH, or $PEG_{20k}$-GH (50 μg/mL based on GH in 1.0 mL medium) and cultured in a

24-well plate for 12 h. The cells were then washed twice with PBS and resuspended in 500 μL PBS for flow cytometry analysis.

For the activation analysis, BMDCs were incubated with $L_{20k}$-, $DL_{20k}$-, or $PEG_{20k}$-GH (50 μg/mL based on GH in 1.0 mL medium) and cultured in a 24-well plate for 24 h. To determine the activation of DCs, cell culture supernatants were collected and the cytokine secretion was analyzed using a CBA Mouse Inflammation Kit. The following FACS analysis was performed according to the manufacturer's instructions.

**Supporting Figures and Tables**
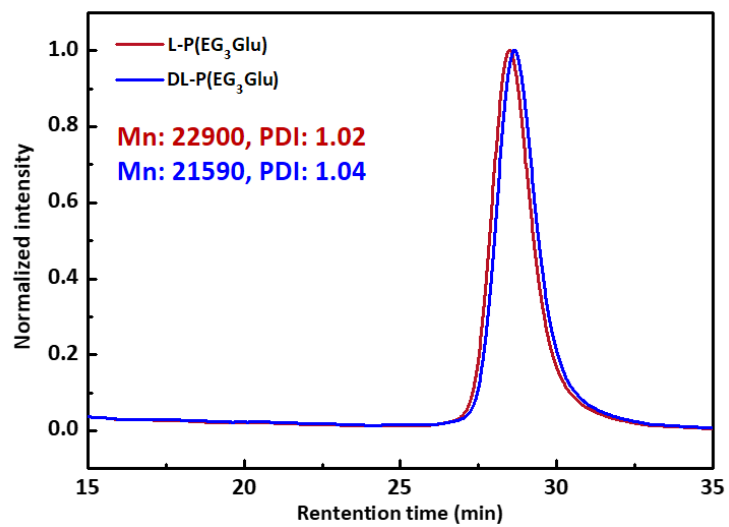


**Figure S1.** GPC curves of L- and DL-P(EG₃Glu).
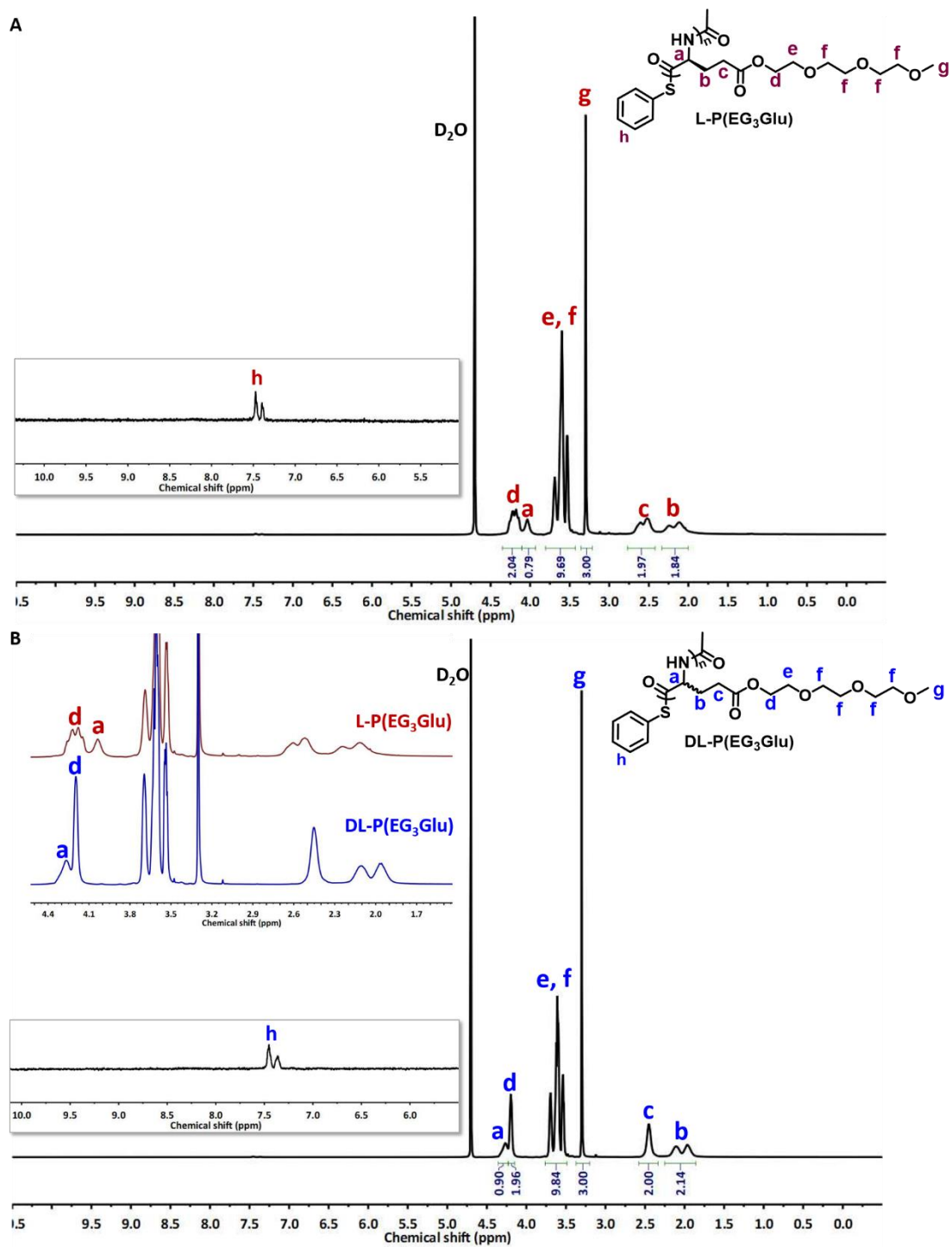
**Figure S2** $^1$H NMR spectra of L-P(EG$_3$Glu) (A) and DL-P(EG$_3$Glu) (B) in D$_2$O.
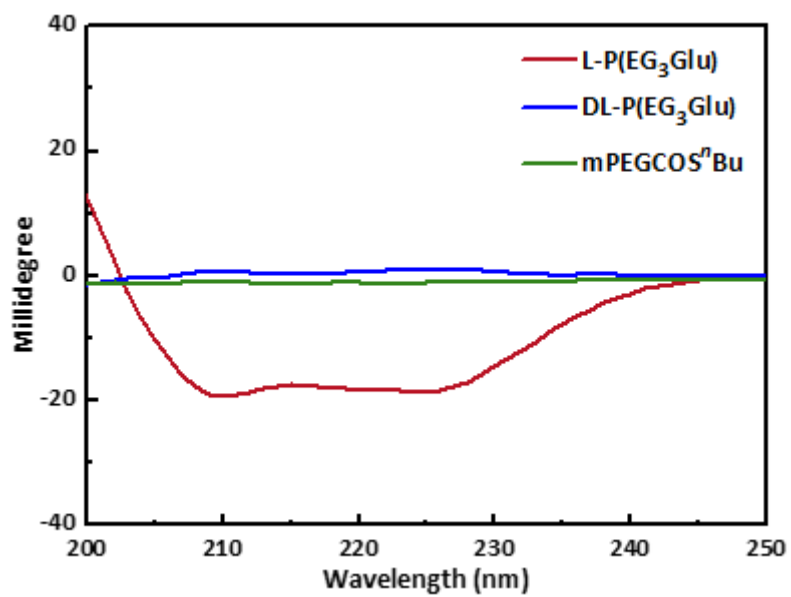
**Figure S3.** CD spectra of L-, DL-P(EG$_3$Glu) or mPEGCOS$^n$Bu at 0.15 mg/mL.
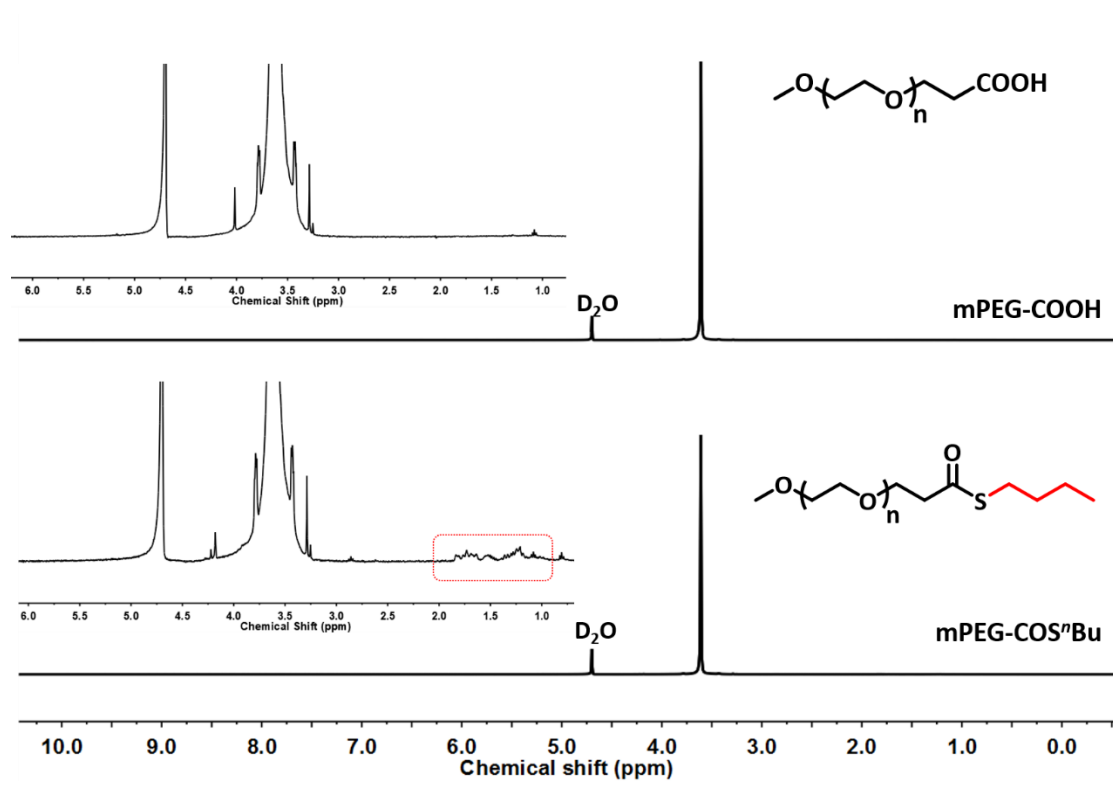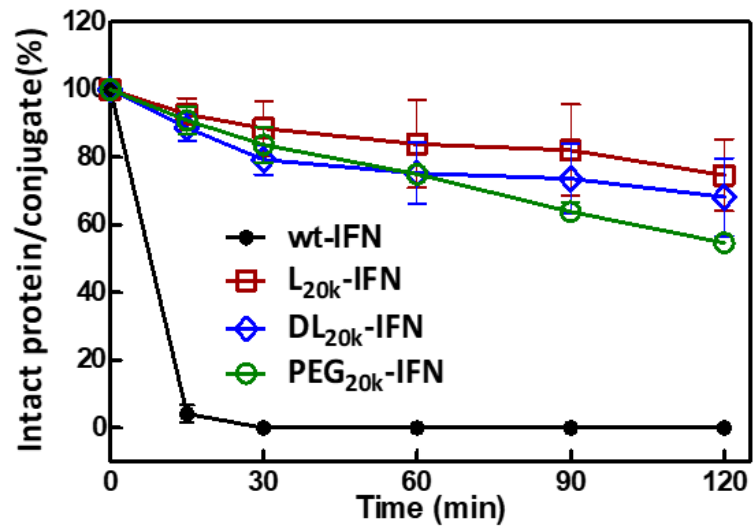
**Figure S4** $^1$H NMR spectra of mPEG-COOH and mPEG-COS$^n$Bu in D$_2$O.

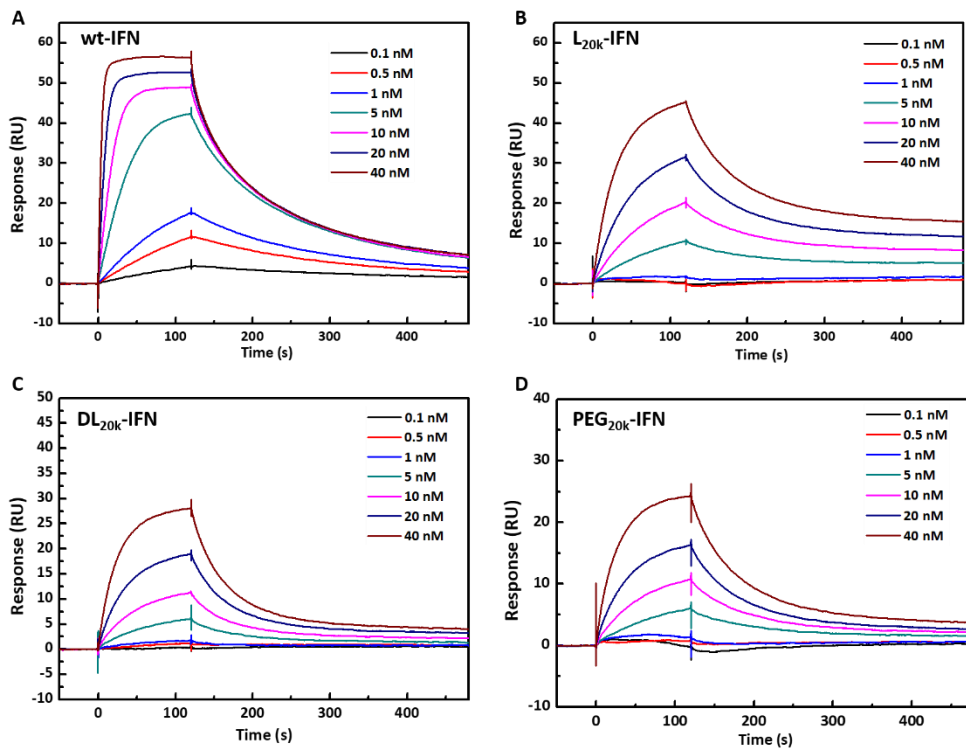**Figure S5.** Trypsin degradation curves of wt-, $L_{20k}$-, $DL_{20k}$- and $PEG_{20k}$-IFN. Data are expressed as mean ± S.D.

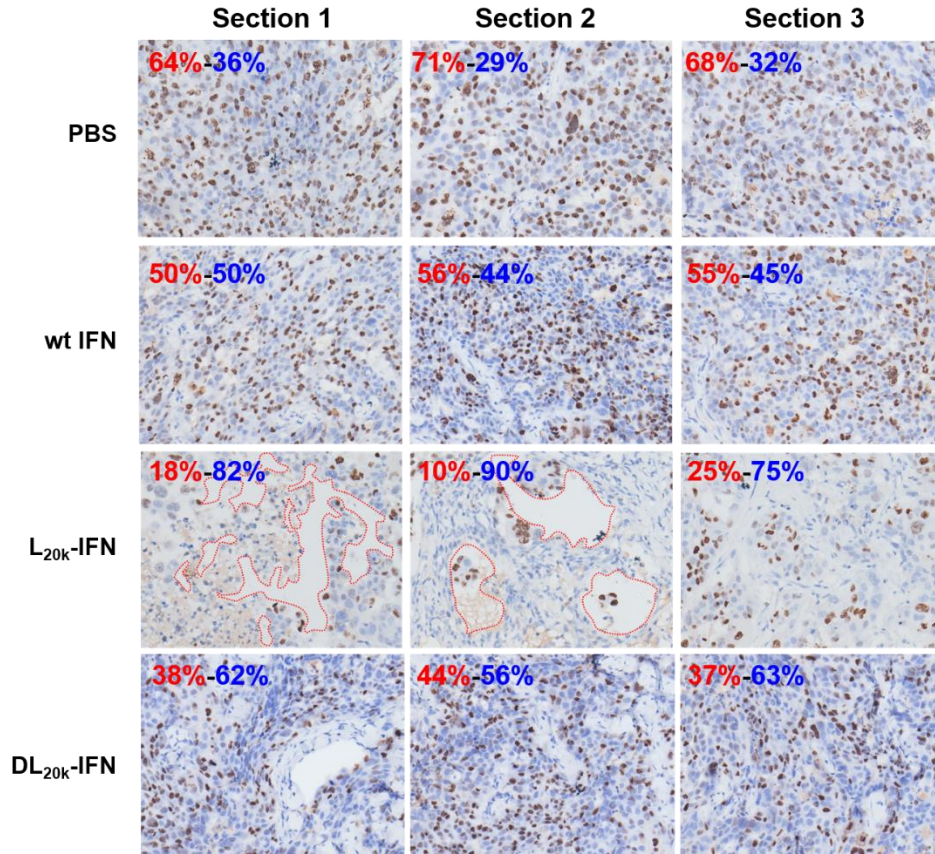**Figure S6.** IFN-IFNAR2 binding curves of wt-IFN (A), $L_{20k}$-IFN (B), $DL_{20k}$-IFN (C) and $PEG_{20K}$-IFN (D).

.

**Figure S7.** Representative photos of the Ki67-stained tumors distracted from the mice receiving PBS saline, wt-, $L_{20k}$-, or $DL_{20k}$-IFN. The red dotted line indicates representative areas of damaged tumor tissues. Section 1-3 represents different tumor locations. Numbers in the images represent the ratio of Ki67 positive (red)/negative (blue) cells.
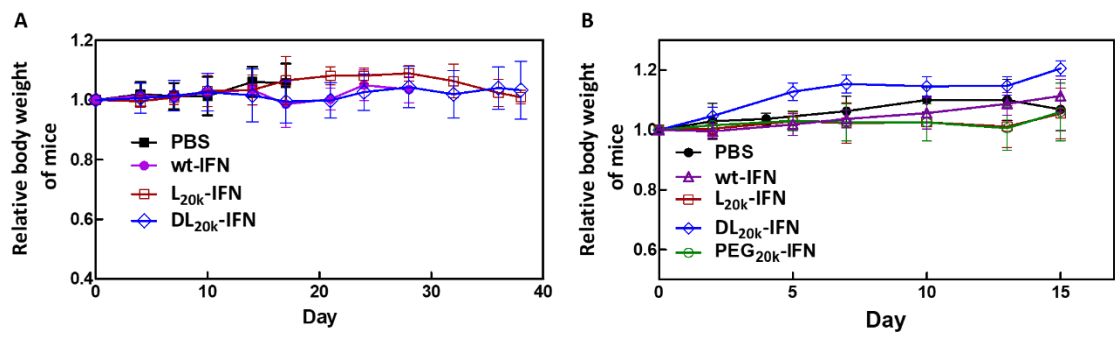
.

**Figure S8.** Relative body weight of BALB/C-nu mice bearing s.c. OVCAR-3 tumors (A) or patient derived xenografted prostate tumor (B). Data are expressed as mean ± S.D.
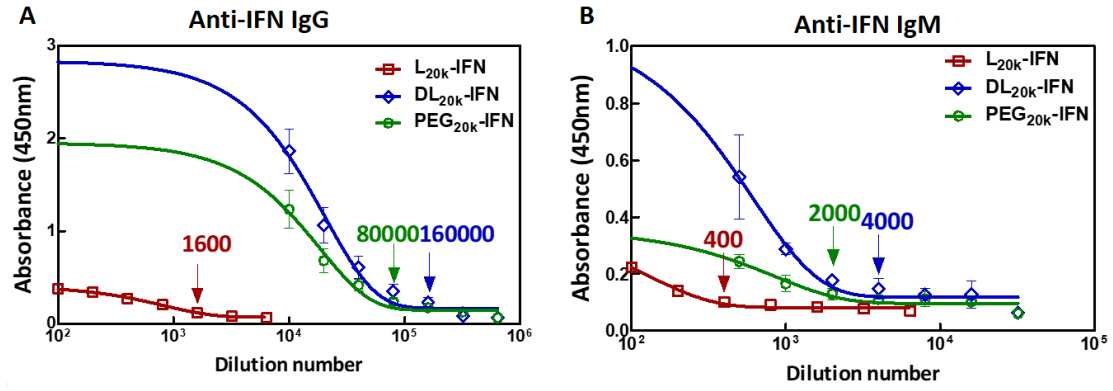
**Figure S9** Anti-IFN IgG (A) and IgM (B) titers in the IFN conjugates immunized sera (week 4), determined by serial dilution ELISA assays. Data are expressed as mean ± S.D.
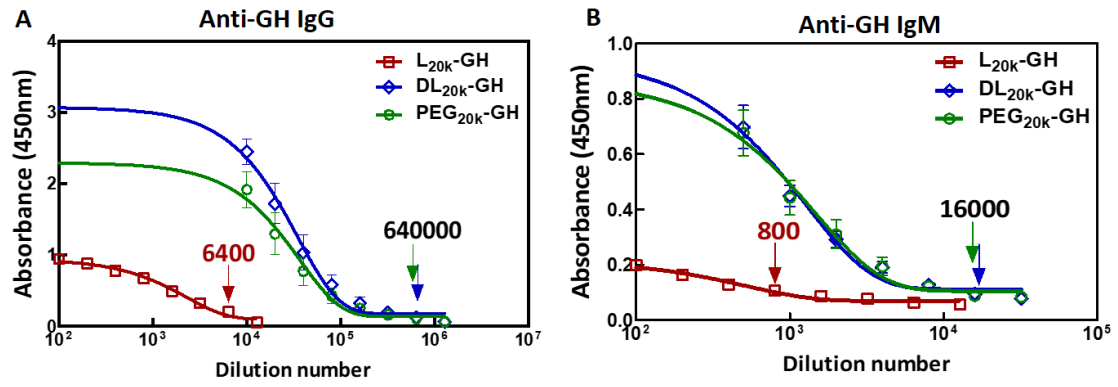
**Figure S10** Anti-GH IgG (A) and IgM (B) titers in the GH conjugates immunized sera (week 4), determined by serial dilution ELISA assays. Data are expressed as mean ± S.D.
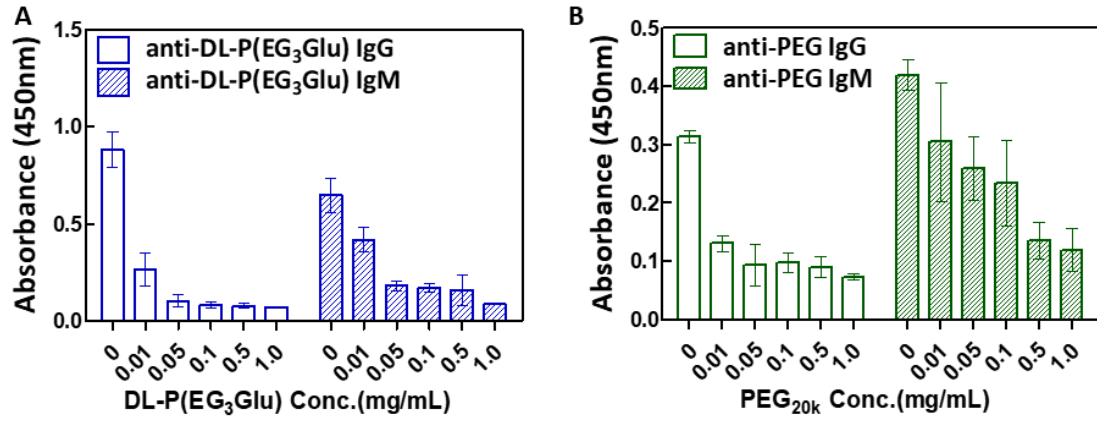
**Figure S11**. Anti-polymer ELISA assays using free DL-P(EG$_3$Glu) (**A**) or PEG (**B**) as the competition agent; the plates were coated with DL$_{20k}$-IFN or PEG$_{20k}$-IFN; sera immunized with DL$_{20k}$-GH or PEG$_{20k}$-GH (week 4) were pre-diluted 200-fold and incubated with the corresponding free polymer at gradient concentrations.

**Table S1** $AUC_{0-24h}$ of GH-polymer conjugates at the 1st and the 3rd dose

| Sample | | $L_{20k}$-GH | $DL_{20k}$-GH | $PEG_{20k}$-GH |
|---|---|---|---|---|
| **$AUC_{0-24h}$ ((ng/mL)*h)** | 1st dose | $76 \pm 12$ | $56 \pm 6$ | $56 \pm 2$ |
| | 3rd dose | $85 \pm 26$ | $3.7 \pm 1.4$ | $3.5 \pm 1.6$ |
| | 3rd/1st dose | 111.8% | 6.6% | 6.2% |

Data are expressed as mean $\pm$ S.D.

## References

1.   Hou, Y.; Yuan, J.; Zhou, Y.; Yu, J.; Lu, H., A concise approach to site-Specific topological protein-poly(amino acid) conjugates enabled by in-situ generated functionalities. *J. Am. Chem. Soc.* **2016,** 138, (34), 10995-11000.

2.   Chen, C. Y.; Wang, Z. H.; Li, Z. B., Thermoresponsive polypeptides from pegylated poly-L-glutamates. *Biomacromolecules* **2011,** 12, (8), 2859-2863.

3.   Liu, S. Y.; Wei, W.; Yue, H.; Ni, D. Z.; Yue, Z. G.; Wang, S.; Fu, Q.; Wang, Y. Q.; Ma, G. H.; Su, Z. G., Nanoparticles-based multi-adjuvant whole cell tumor vaccine for cancer immunotherapy. *Biomaterials* **2013,** 34, (33), 8291-8300.