Supplementary Material

Increasing lean muscle mass in mice via nanoparticle-mediated hepatic delivery of follistatin mRNA

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Materials

γ-benzyl L-glutamate (BLG) was purchased from Alfa Aesar (Tewksbury, MA). α-Aminopropyl-ω-aminopropoxy-polyoxyethylene (NH2-PEG-NH2) (Mn = 2000 g/mol, PDI = 1.03) was purchased from NOF America Corporation (White Plains, NY. All other reagents were of analytical grade and purchased from VWR International, LLC (Radnor, PA) or Fisher Scientific Inc. (Fairlawn, NJ).

Synthesis and characterization of N-substituted polyethylene glycol-diblock-polyglutamide (PEG[Glu(DET)]$_2$). The schematic for the synthesis is presented in (Figure S1). Step one is the synthesis of γ-benzyl L-glutamate N-carboxyanhydride (BLG-NCA) using triphosgene. Followed by step two, which is the polymerization of BLG-NCA using NH_2-PEG-NH_2 (M_w = 2000) as an initiator. Finally, step three is the replacement of the benzyl sidechain by diethylenetriamine. BLG-NCA are synthesized according to the Fuchs-Farthing method.[1] Briefly, 10 g of BLG (42.5 mmol) is dried under vacuum for 3 h and is mixed with 0.4 equivalence of triphosgene (5 g for BLG). The dried mixture is then suspended in 60 ml of anhydrous THF with stirring at 40°C. The entire reaction is performed under an argon atmosphere for at least 4 h. The formation of BLA-NCA and BLG-NCA (1) is indicated by the formation of a clear pale-yellow solution. Then the solution was cooled to room temperature followed by stepwise addition of 150 ml anhydrous hexane. The solution is stored at -20°C overnight to facilitate the precipitation of white NCA crystal which is then purified by recrystallization from THF and hexane.

The synthesis of tri-block PBLG copolymers using NH_2-PEG-NH_2 (2) as initiators is based on a previously described method. [2, 3] The BLG-NCA are polymerized by ring opening initiated by the terminal primary amine groups of NH_2-PEG-NH_2 (Figure S1). The process starts with freeze-drying of the initiator, NH_2-PEG-NH_2 (0.1 g, 0.05 mmol), from benzene, followed by the addition of 1 ml anhydrous DMF. Freshly prepared BLG-NCA (1.31 g, 5 mmol) is dissolved in 10 ml anhydrous DMF and transferred to the initiator solution. The reactions are carried out at 45°C under argon atmosphere for 48 hr. The resulting polymer PEG- $(pGlu)_2$ (3) (Figure S1) is precipitated in cold diethyl ether then freeze-dried from benzene.

The last step in the synthesis scheme is the replacement of the benzyl sidechain with diethylenetriamine (DET) (4) by the aminolysis reaction (Figure S1). Polymer from (3) is dissolved in anhydrous DMF (100 mg/ml) followed by the addition of DET (20 equivalence of the benzyl repeating unit). The reaction is run at room temperature for 48 hr. Upon completion, the polymer solution is dialyzed against 0.1 M HCl for 24 h, then against 0.01 M HCl for 24 h, and finally against ddH_2O for 24 h using regenerated cellulose dialysis tubing (MWCO 5000). The polymer PEG[pGlu(DET)]$_2$ (5) (Figure S3) is collected by freeze-drying to remove water.

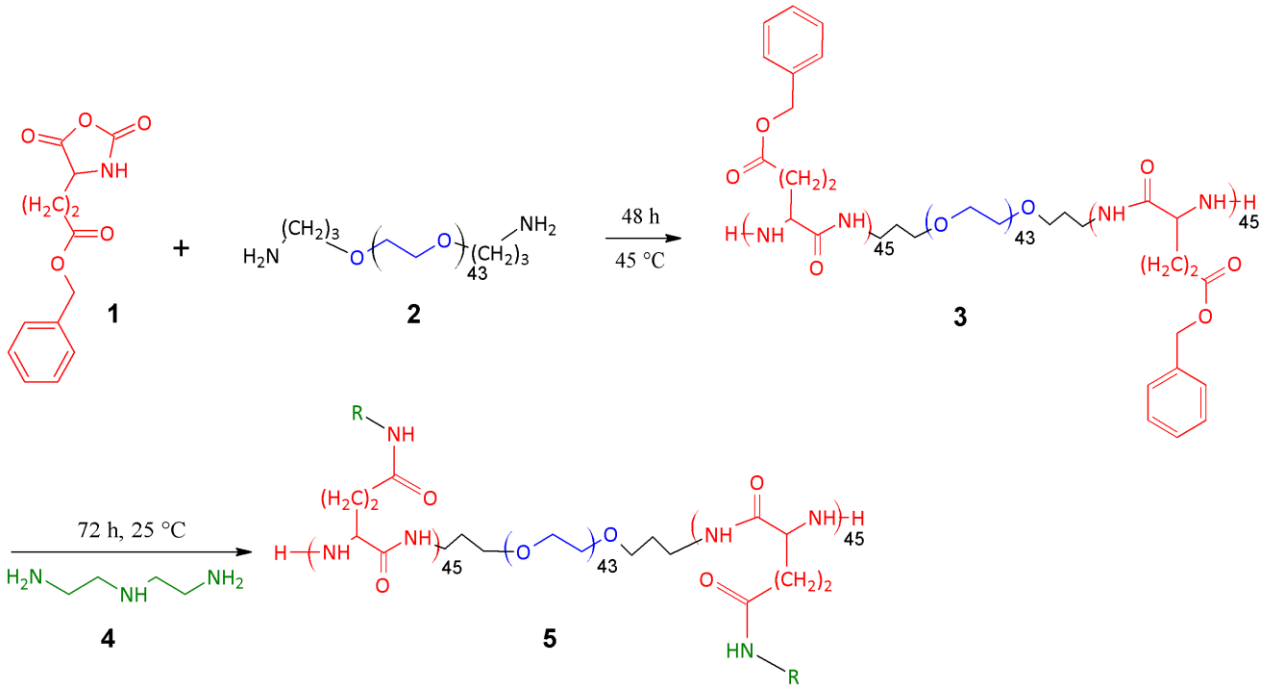

Figure S1. Synthesis scheme for N-substituted polyethylene glycol-diblock- polyglutamide (PEG[pGlu(DET)]$_2$).

The ^{1}H NMR spectra of the synthesized polymer are recorded using a Bruker 400 MHz Advance III spectrometer using deuterated DMSO. The FTIR spectra of synthesized polymers are obtained using a Nicolet-100 Infrared Spectrophotometer. The resolution is set at 4 sec^{-1}, and 16 scans per sample. The samples are dissolved in methylene chloride and cast on KBr plates. The molecular weight and chemical composition of PEG- (pGlu)$_2$ (3) are determined from the resulting NMR spectra. The number of the γ-benzyl L-glutamate repeating units is determined to

be 84, and the polymer molecular weight (*Mn*) is 17,877 Da) (Figure S2). The aminolysis reaction and the formation of (PEG[pGlu(DET)]$_2$) (5) is confirmed by NMR and FTIR (Figure S3 and S4), and the molecular weight is calculated to be 19,981 Da.

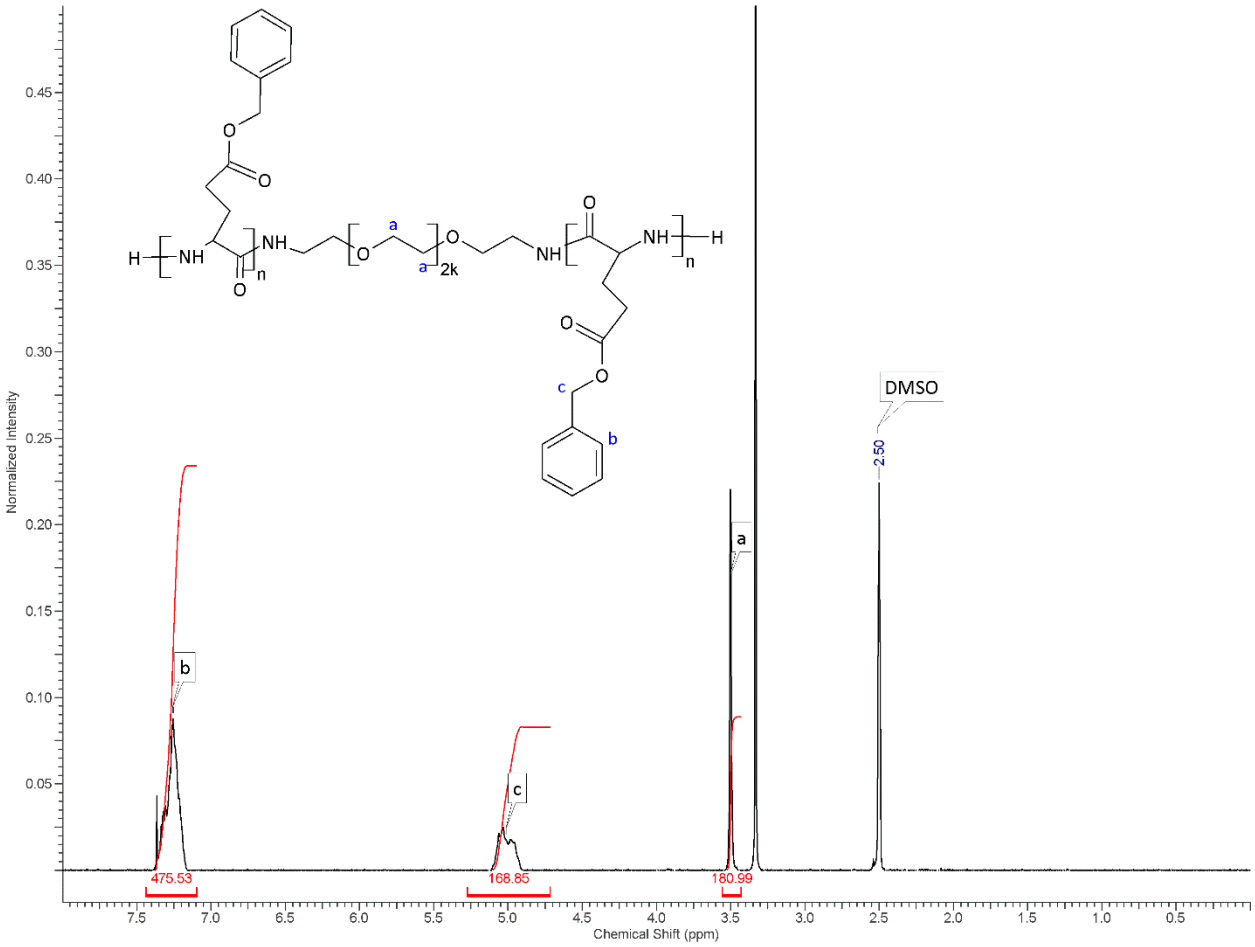

Figure S2. ^{1}H-NMR spectra of PEG- $(pGlu)_2$ (3). NMR measurements are performed on a Bruker DPX-400 NMR spectrometer at 400 MHz in DMSO-d_6 and at 80 °C.

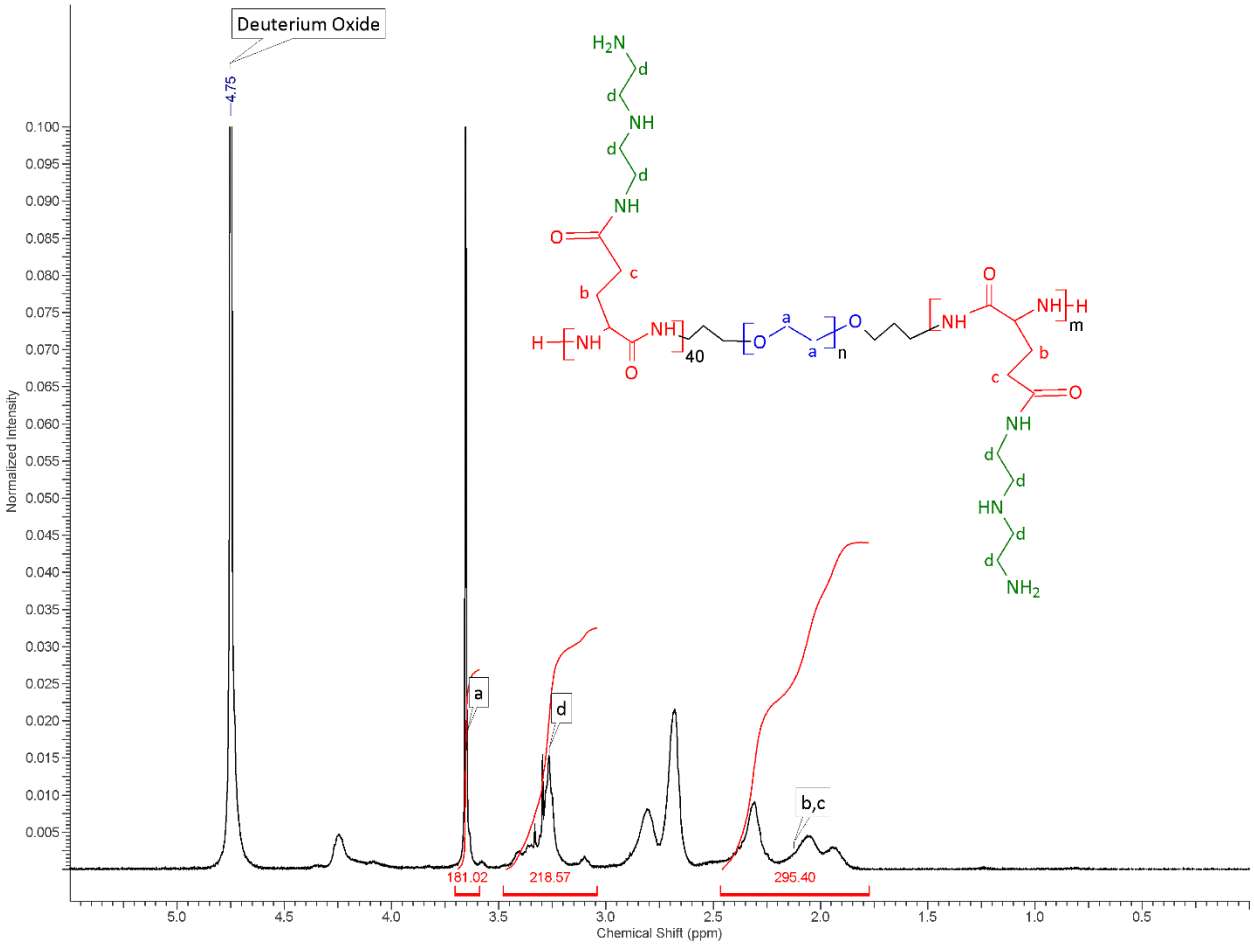

Figure S3. ^{1}H-NMR spectra of PEG[pGlu(DET)]$_2$ (5). NMR measurements are performed on a Bruker DPX-400 NMR spectrometer at 400 MHz in DMSO-d_6 and 80 °C.

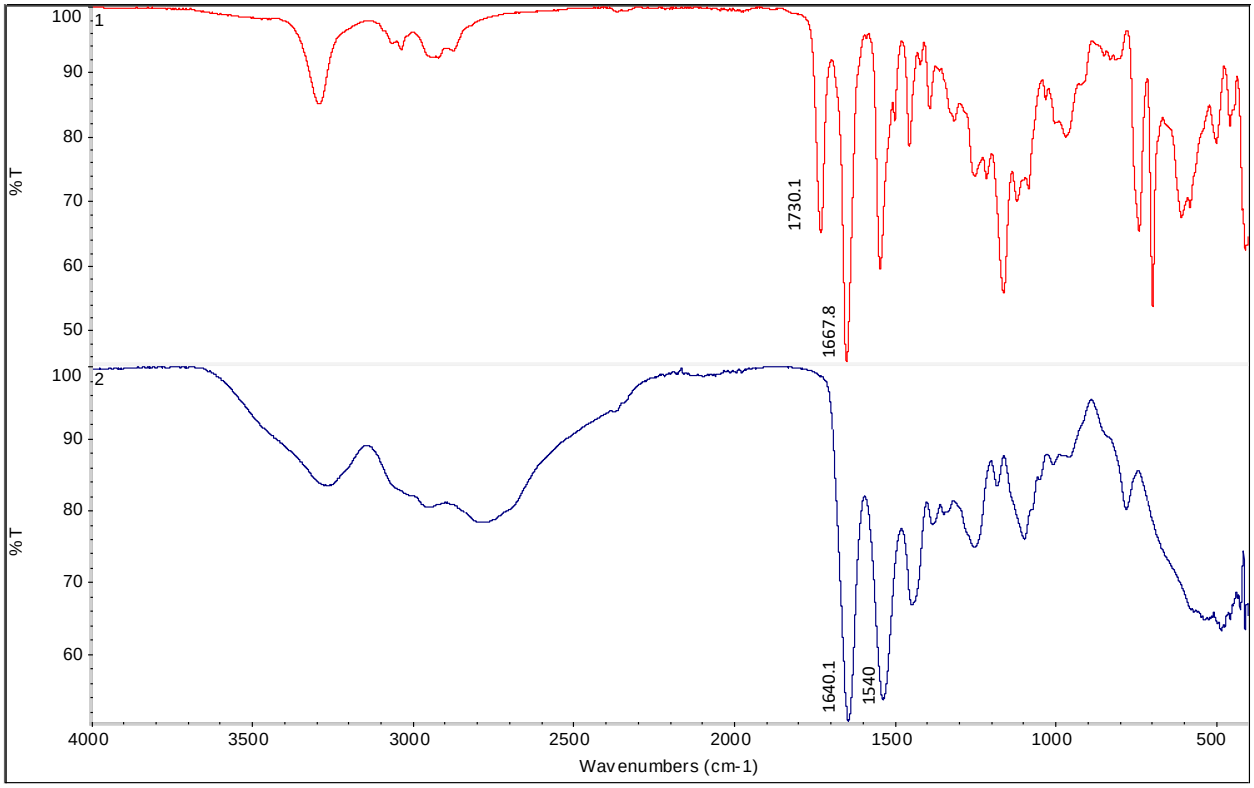

Figure S4. FTIR spectra confirm the reaction completion. The replacement of the ester peak (C=O, 1730 cm^{-1}) by the sharp amide peaks (C=O at 1640 cm^{-1} and N-H at 1540 cm^{-1}) is observed on FTIR spectra

Gel retardation assay. The agarose gel retardation assay was performed to assess the electrostatic complexation of the polymer and mRNA at various N/P ratios by following the previously published procedure.[4] Briefly, mRNA and polymer at the different N/P ratios were mixed in RNAse free milliQ water and vortexed (Eppendorf Mixmate, Hauppauge, NY) at 1500 rpm at room temperature for 30 min prior to addition to the 4% NuSieve agarose gel (Lonza, Rockland ME, USA). After complexation, glycerol gel loading dye (Amresco, Solon, OH) was added to samples with the various N/P ratios (0.5, 1, 2, 4 and 8), then 15 μL of sample was loaded onto the gel and ran for 30 min at 100 V (Bio-Rad power supply, Hercules, CA). The free mRNA bands were visualized using an ultraviolet (UV) light imaging system (UVP BioDoc-It Imaging System, Upland, CA).

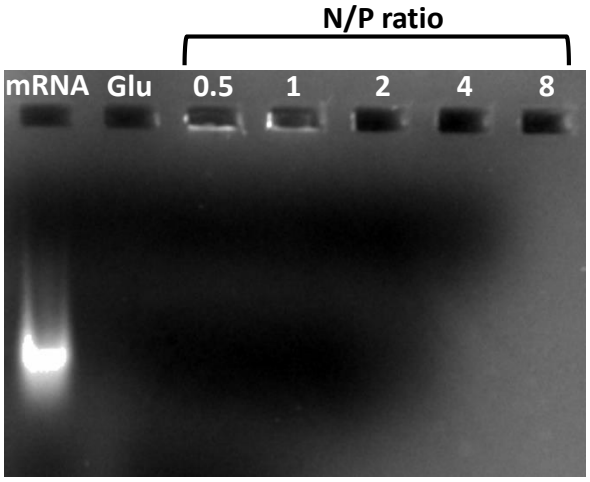

Figure S5. Representative gel electrophoresis image of free mRNA, free N-substituted polyglutamide (PEG[Glu(DET)]$_2$ or Glu) and nanoparticles prepared by mixing mRNA with the employed polymer at N/P ratios of 0.5, 1, 2, 4 and 8. Of note, an N/P ratio does not vary the amount of the mRNA encapsulated into nanoparticles; it only varies the concentration of the polymer used to complex mRNA into nanoparticles. Each well contains the same amount of mRNA. Efficient binding of mRNA molecules with the polymer retards their gel electrophoretic mobility and prevents mRNA staining by ethidium bromide when compared to free mRNA.

Size and zeta potential measurements

The hydrodynamic size, polydispersity index (PDI) and the zeta potential of the prepared nanoparticles were measured by Malvern ZetaSizer NanoSeries (Malvern Instruments, UK) according to the manufacturer's instructions. Prior to measurements, 10 μL of the prepared nanoparticles were diluted with 900 μL of MilliQ water. The intensity of the He−Ne laser (633 nm) was measured at an angle of 173°. All measurements were performed at 25 °C after pre-equilibration for 2 min, and each parameter was measured in triplicate.

Transmission Electron Microscopy (TEM):

The size and morphology of the synthesized nanoparticles were evaluated using Talos Arctica microscope (FEI) transmission electron microscope. 4 μl of nanoparticle sample was applied onto a glow-discharged (15mA 60sec.) 400 mesh copper grid with thin carbon film supported by lacey carbon substrate (Ted Pella 01824). Grids were blotted for 3 sec at 22 °C and 100% humidity using a blot force of -15, then plunged into liquid ethane using a Vitrobot Mark IV (FEI). Images were recorded with a K2 Summit camera (Gatan) in counting mode on a Talos Arctica microscope (FEI) operated at 200 kV. Cryo-EM images were collected with a defocus range of 2-4 μm.

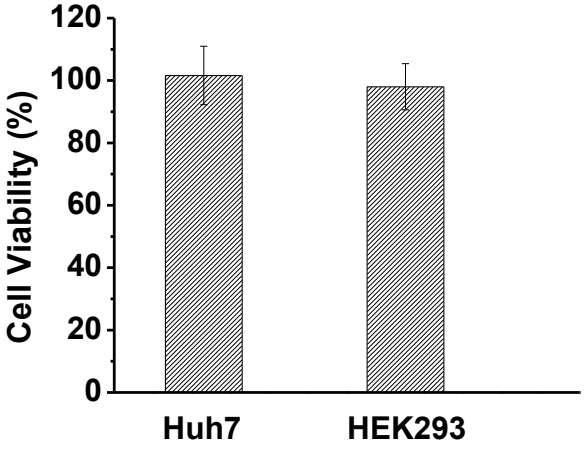

Figure S6. Viability of Huh7 human liver cells and HEK293 human embryonic kidney cells treated for 48 h with PEG-modified mRNA nanoparticles prepared at the N/P ratio of 2.

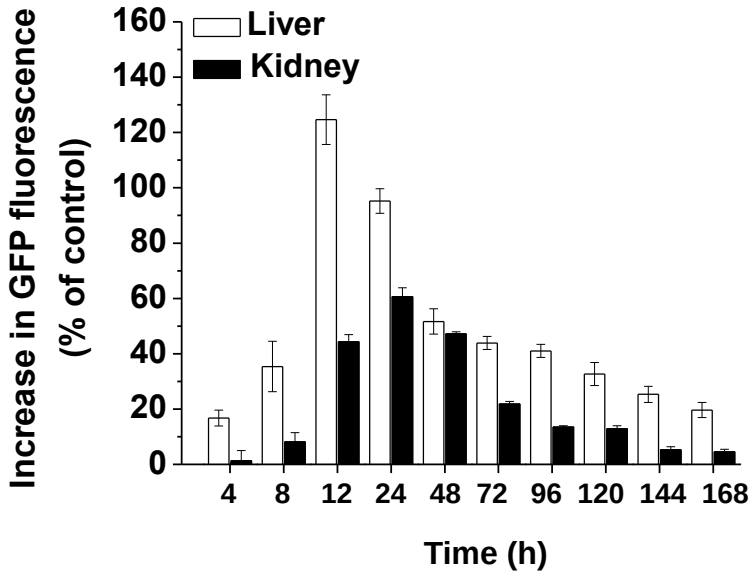

Figure S7. The percent change in mean fluorescence intensity generated by GFP in livers and kidneys at various time points after a single subcutaneous injection of the nanoparticles containing GFP mRNA. The livers and kidneys were dissected from mice pre-injection and at various time points post-injection and imaged using an IVIS Lumina XRMS In Vivo Imaging System. The regions of interest were drawn over the livers and kidneys of the fluorescence images, and the green fluorescence signal for each organ was measured using IVIS Living Image™ software. The percent change in mean fluorescence intensity was calculated as the following:

% change = (Fl (treated) – Fl (control))/ Fl (control) *100, where

Fl (treated) – green fluorescence intensity in organs at various time points after a single subcutaneous injection of the nanoparticles containing GFP mRNA.

Fl (control) - green fluorescence intensity in organs dissected from non-treated mice

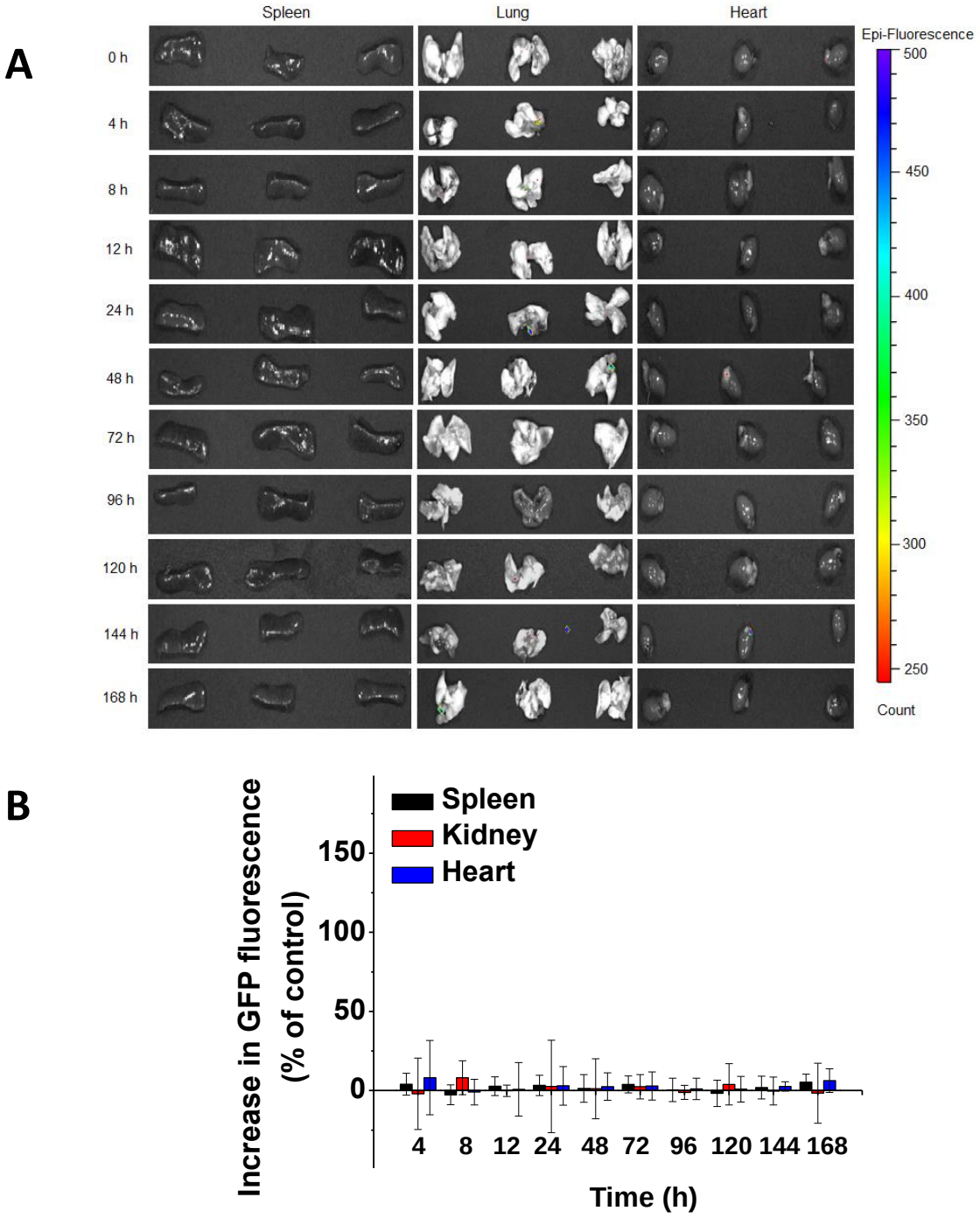

Figure S8. (A) GFP production in the spleens, lungs, and hearts at various time points following subcutaneous injection of the nanoparticles loaded with GFP mRNA at a dose of 0.5 mg/kg. **(B)** The percent change in mean fluorescence intensity generated by GFP in the spleens, lungs, and

hearts at various time points after a single subcutaneous injection of the nanoparticles containing GFP mRNA.

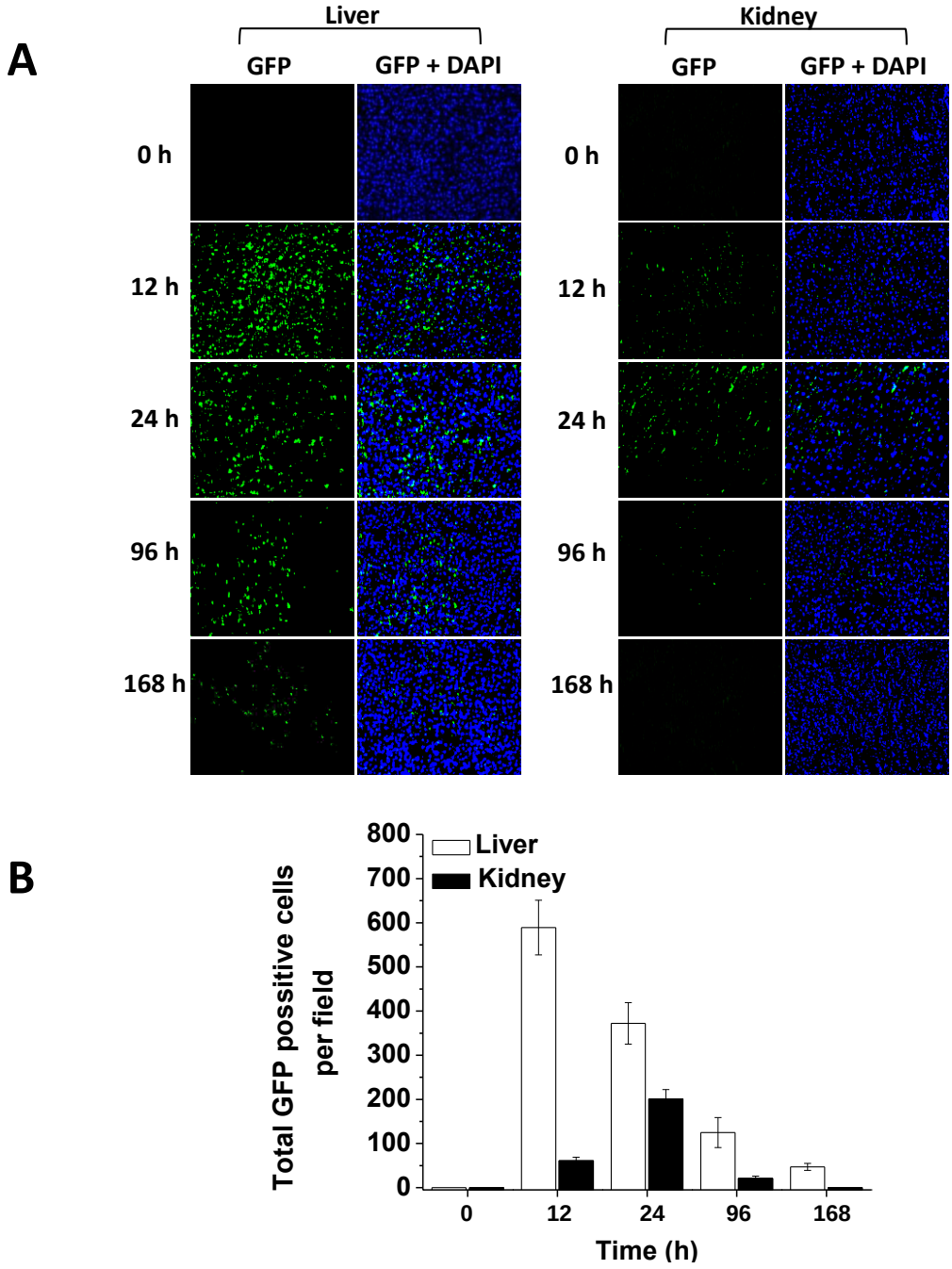

Figure S9. (A) Representative fluorescence images of the liver and kidney cross-sections harvested from the mice at the various time points following subcutaneous injection of the nanoparticles loaded with GFP mRNA at a dose of 0.5 mg/kg. Green and blue colors represent

fluorescence signals generated by the expressed GFP and DAPI-stained nuclei, respectively. **(B)** Mean numbers of GFP-positive cells per field of liver and kidney sections.

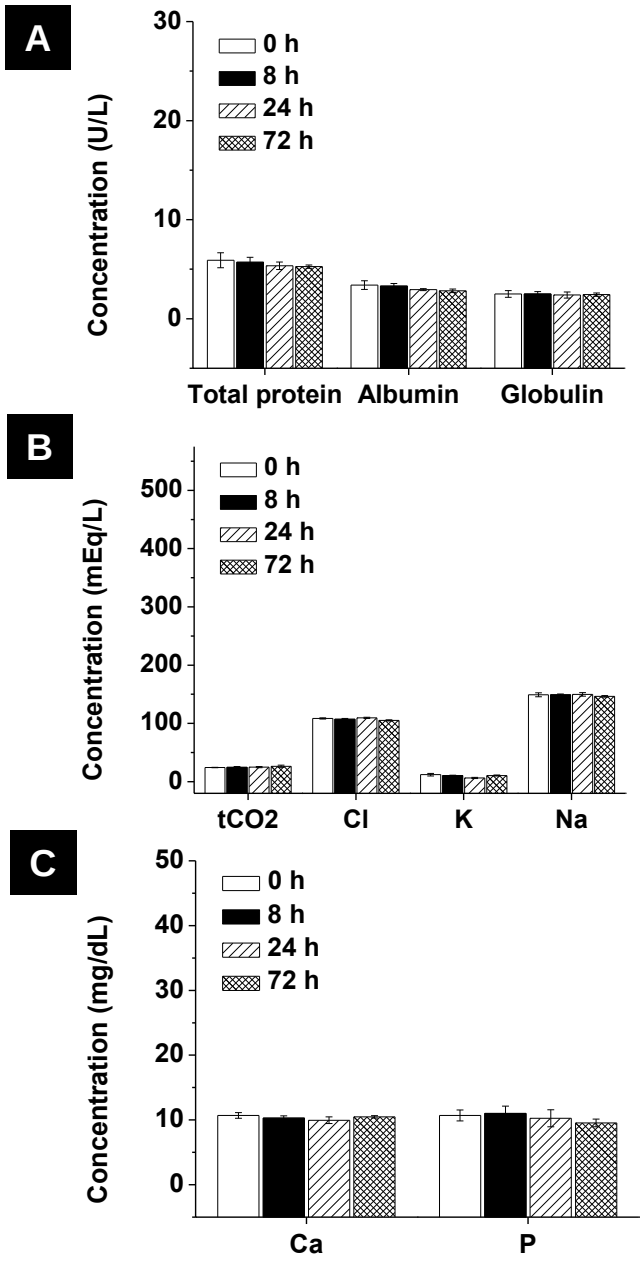

Figure S10. The blood levels of **(A)** total protein, albumin and globulin and **(B)** - **(C)** total carbon dioxide (tCO2) and electrolytes (chloride (Cl), potassium (K), sodium (Na), calcium (Ca),

phosphorus (P)) in non-treated mice (0 h) and mice subcutaneously injected with the FS-344 mRNA loaded nanoparticles (0.5 mg/kg).

References

1. Daly, W. H.; Poché, D. The Preparation of N-Carboxyanhydrides of A-Amino Acids Using Bis(Trichloromethyl)Carbonate. *Tetrahedron Lett.* 1988, *29*, 5859–62.
2. Itaka, K.; Ishii, T.; Hasegawa, Y.; Kataoka, K. Biodegradable Polyamino Acid-Based Polycations as Safe and Effective Gene Carrier Minimizing Cumulative Toxicity. *Biomaterials* 2010, *31*, 3707-14.
3. Rao, D. A.; Mishra, G.; Doddapaneni, B. S.; Kyryachenko, S.; Wierzbicki, I. H.; Ngyuen, D. X.; Shah, V.; Al Fatease, A. M.; Alany, R. G.; Alani, A. W. G. Combinatorial Polymeric Conjugated Micelles with Dual Cytotoxic and Antiangiogenic Effects for the Treatment of Ovarian Cancer. *Chemistry of Materials* 2016, *28*, 6068−6079.
4. Schumann, C.; Taratula, O.; Khalimonchuk, O.; Palmer, A. L.; Cronk, L. M.; Jones, C. V.; Escalante, C. A. Ros-Induced Nanotherapeutic Approach for Ovarian Cancer Treatment Based on the Combinatorial Effect of Photodynamic Therapy and Dj-1 Gene Suppression. *Nanomedicine-Uk* 2015, *11*, 1961-70.