Supplementary Information

Facile preparation, structural modulation, and bone-targeting of single-chain polypeptide nanoparticles

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Materials, cells, and animals

All chemicals were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and used without further purification unless otherwise specified. Deuterated solvents were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, USA). Organic solvents, propylene oxide (PO), trifluoroacetic acid (TFA), and *n*-hexylamine were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HA nanoparticles were obtained from Aladdin Scientific (Shanghai, China). Dialysis bags with molecular weight cut-off (MWCO) of 1.0 and 3.5 kDa were purchased from Yuanye Bio-Technology (Shanghai, China). A CCK-8 assay kit was purchased from ApexBio (Suzhou, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (NY, USA).

Anhydrous dichloromethane (DCM), tetrahydrofuran (THF) and hexane were dried using alumina columns. Anhydrous *N*,*N*-dimethylformamide (DMF) was pre-treated with polymer-bound isocyanate (MilliporeSigma, St. Louis, USA) to remove any amino residues. Ketal-based diacrylate crosslinker, propane-2,2-diylbis(oxy)bis(ethane-2,1-diyl) diacrylate (KTDA), was prepared according to literature procedures.[1] The monomer N^{ε}-*tert*-butoxycarbonyl-$_{L}$-lysine *N*-carboxyanhydride (BLL-NCA) was synthesized following literature procedures.[2]

RAW 264.7 cells (mouse monocyte macrophages) were a gift from the School of Pharmacy, Soochow University. MC3T3-E1 cells (murine osteoblast precursor cells) were purchased from Shanghai FuHeng Biology Co. Ltd. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) or minimum essential medium α (αMEM) supplemented with 1% nonessential amino acids, 10% FBS, 1% $_{L}$-glutamine, and a penicillin–streptomycin cocktail.

Female BALB/c mice (4-6 weeks old) were purchased from Cavens Model Animal Co., Ltd. (Changzhou, China) and housed in a specific pathogen-free (SPF) room at 25 ^{o}C. All animal procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) and were approved by the Animal Ethics Committee of Soochow University.

Instruments

^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE NEO-400 MHz spectrometer. The chemical shifts (δ) were referenced to the residual protons in the deuterated solvents. MestReNova software (version 6.1.0, Mestrelab Research, Escondido, USA) was used for all NMR analyses. Diffusion ordered NMR spectroscopy (DOSY) experiments were performed on a Bruker AVANCE NEO-400 MHz spectrometer equipped with a Z-axis gradient coil (maximum gradient strength 53.5 G/cm) at 25 ºC. DOSY data were collected using the ledbpgp2s pulse sequence with gradient strengths linearly increasing from 2% to 95% in 16 steps. The gradient pulse duration (δ) was set to 5 ms, and the diffusion delay time (Δ) was 200 ms. The diffusion coefficient (D) of the characteristic peak was fitted using an exponential decay model. Gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (1260 Infinity II, Agilent, Santa Clara, USA) and a multi-angle static light scattering (MALS) detector (DAWN, Wyatt Technology, Santa Barbara, USA). The detection wavelength of the MALS detector was set at 658 nm. Separations were performed using serially connected size exclusion columns (KD-803, KD-804, and KD-806, 8 × 300 mm, Shodex, Yokohama, Japan) using DMF containing LiBr (0.1 mol L^{-1}) as the mobile phase at a flow rate of 1 mL min^{-1} at 60 ºC. The MALS detector was calibrated using pure toluene and was used for the determination of absolute MWs. The MWs of polymers were determined based on the dn/dc value of each polymer sample calculated offline by using the internal calibration system processed by the ASTRA 8 software (version 8.1.0, Wyatt Technology, Santa Barbara, USA). Fourier transform infrared (FTIR) spectra were recorded using a Thermo Fisher Nicolet iS20 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, USA). Circular dichroism (CD) data was collected using a chirascan V100 CD spectrometer (Applied Photophysics, Leatherhead, UK). Dynamic light scattering (DLS) measurements were performed using a Nano ZS90 instrument (Malvern, UK) at 25 ºC. Transmission electron microscopy (TEM) images were collected using a Tecnai G2 F20 field emission transmission electron microscope (FEI, Hillsboro, USA). Cell microplates were analyzed using a BioTek Synergy H1 multifunctional microplate reader (Agilent, Santa Clara,

USA). Flow cytometry experiments were performed using a BD FACSAria III system (FACSAria, Franklin Lakes, USA) and analyzed using FlowJo software (version 10.8.1, BD Biosciences, Franklin Lakes, USA). The fluorescence imaging of collected organs *ex vivo* was performed using the PerkinElmer IVIS optical imaging system (PE, USA). The pH of solutions was measured using a PHS-3C pH meter (INESA, Shanghai, China).

Supplementary Figures

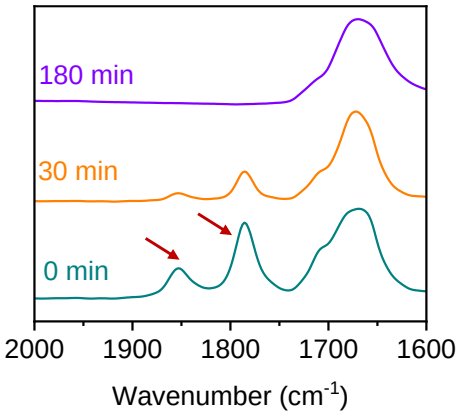

Figure S1. Overlaid FTIR spectra of polymerization mixture at different time points. The anhydsride peaks at 1785 and 1853 cm^{-1} were highlighted with red arrows.

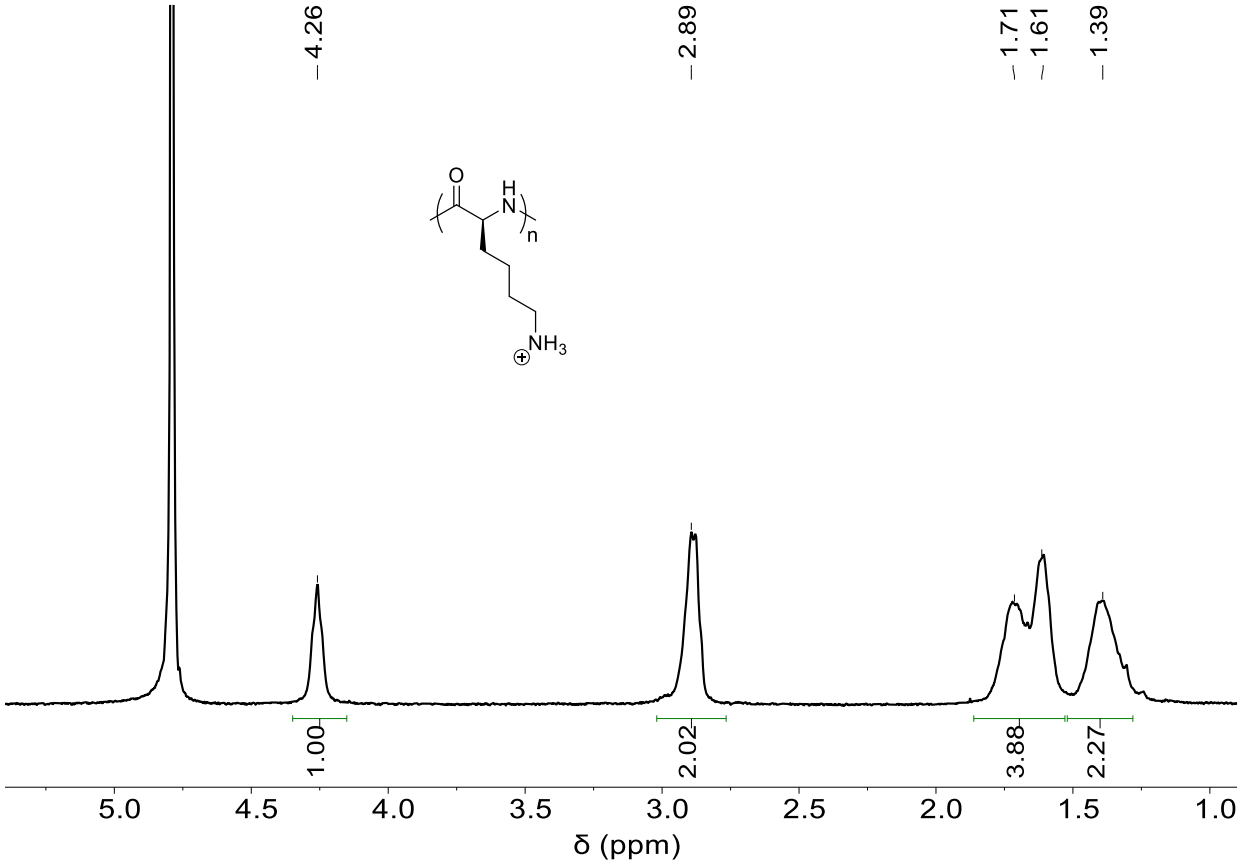

Figure S2. ^{1}H NMR of PLL_{200} in D_2O (400 MHz).

The deprotection efficiency was calculated from the integral ratio of residual *tert*-butoxycarbonyl protons (buried in the peak at 1.39 ppm, which required subtraction of the methylene protons on PLL side chains) to methylene protons adjacent to amino groups (i.e., 2.89 ppm).

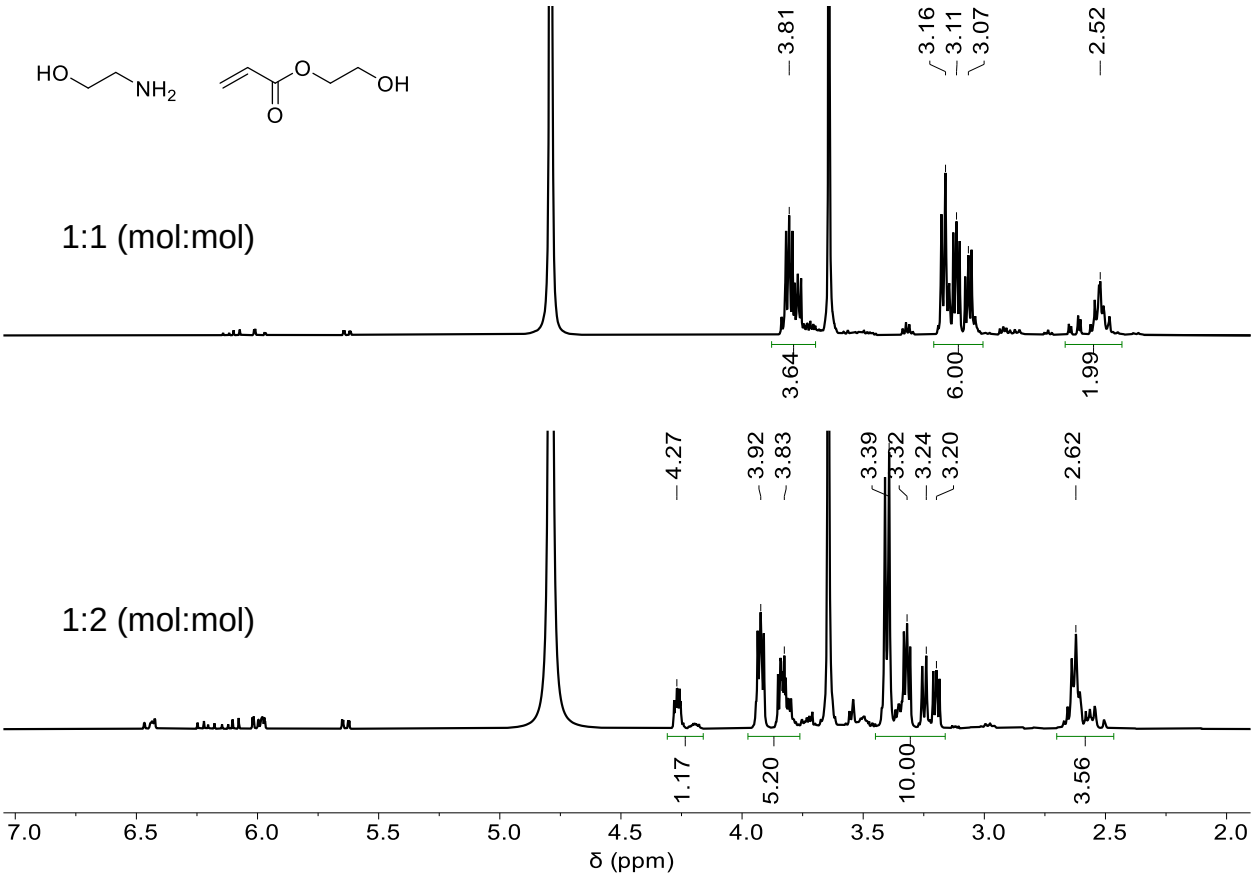

Figure S3. Overlaid ^{1}H NMR spectra showing the reaction product of small-molecular model compounds, ethanolamine and 2-hydroxyethyl acrylate, at different feeding ratios at 50 ^{o}C at pH 9.5 in D_2O.

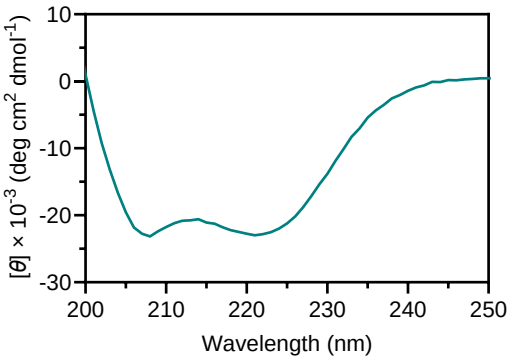

Figure S4. CD spectrum of PLL_{200} in water at pH 9.5.

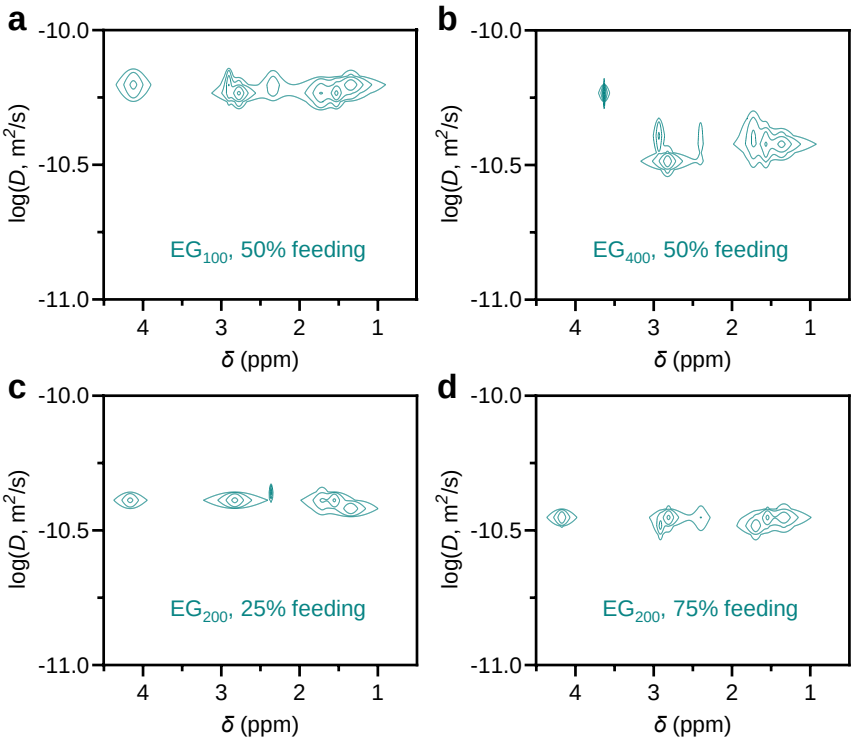

Figure S5. Summary of DOSY NMR spectra of single-chain polypeptide nanoparticles from PLL with different chain lengths (a, b) or at different crosslinking feeding (c, d).

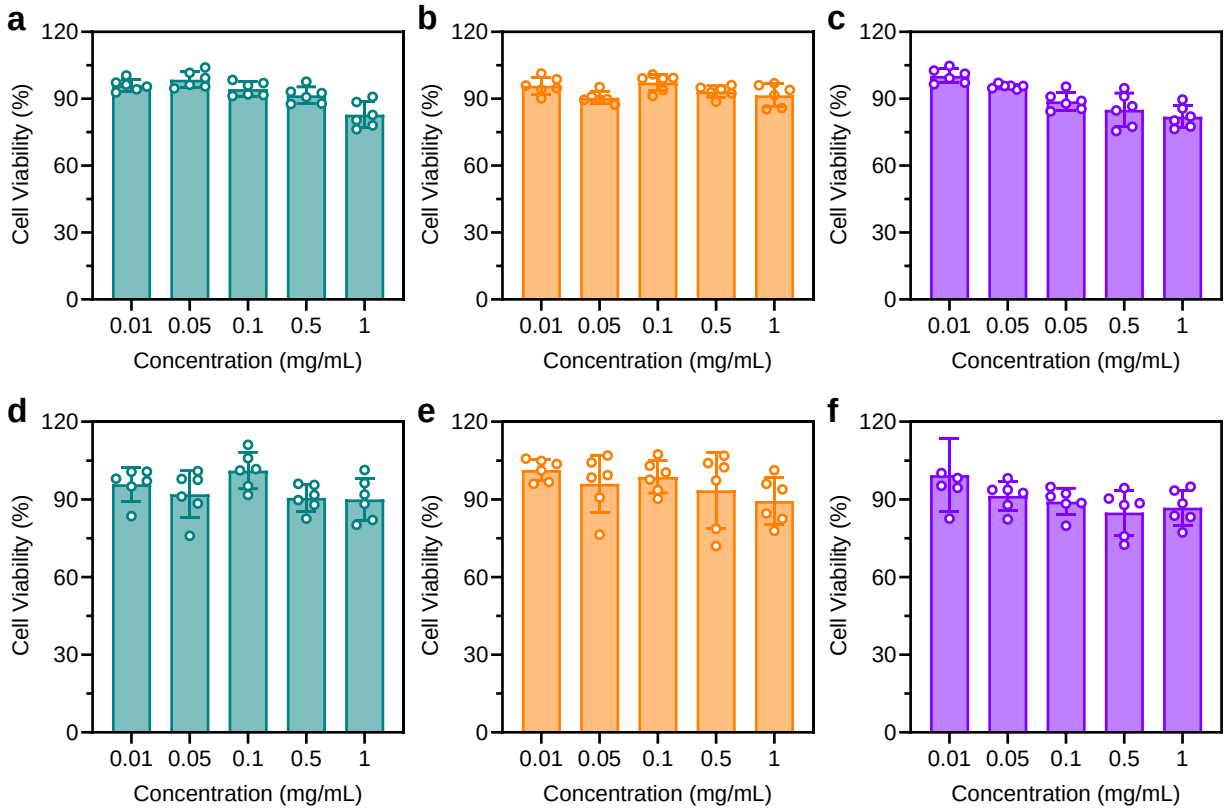

Figure S6. Cell viability of RAW 264.7 cells (a-c) and MC3T3-E1 cells (d-f) after 24-h incubation with anionic, single-chain polypeptide nanoparticles, including GA (a and d), SA (b and e), and AA (c and f), at various nanoparticle concentrations (n = 6).

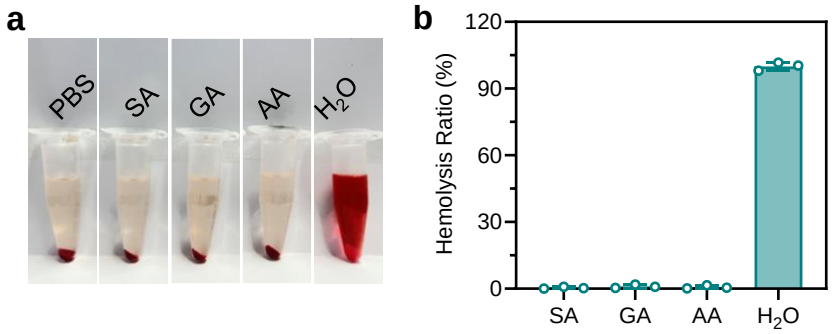

Figure S7. Hemocompatibility of anionic, single-chain polypeptide nanoparticles. Photographs demonstrating the erythrocyte sedimentation (a) and hemolysis ratio of erythrocyte (b) after treatment with SA, GA, or AA (n = 3). H_2O and PBS were used as positive and negative controls, respectively.

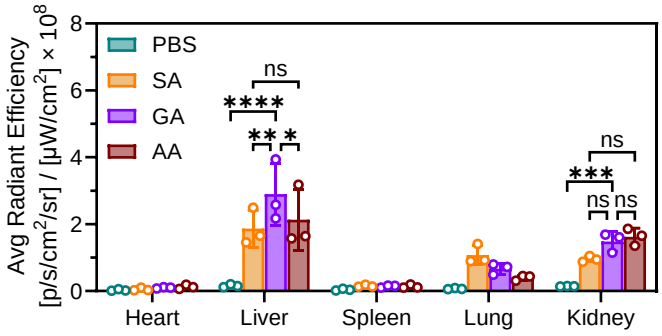

Figure S8. Semi-quantitative analysis of average radiant efficiency of major organs (i.e., heart, liver, spleen, lung, and kidney) of mice at 24 h post *i.v.* injection of Cy5-labeled anionic, single-chain polypeptide nanoparticles (n = 3). Mice injected with PBS served as control. ns = not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

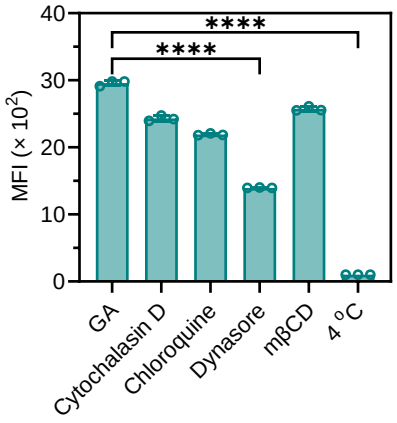

Figure S9. Mean fluorescent intensity of RAW 264.7 cells incubated with Cy5-labeled GA nanoparticles at 37 °C or 4 °C, or pre-treated with various endocytic inhibitors (n = 3). ****p < 0.0001.

Supplementary Tables

Table S1. GPC characterization of PBLL.[a]

Entry	Polymer	$M_{n,\,theo.}$ (kDa)	$M_{n,\,GPC}$ (kDa)[b]	$Đ$[b]	Deprotection efficiency (%)[c]
1	$PBLL_{100}$	22.9	26.0	1.05	94
2	$PBLL_{200}$	45.7	42.9	1.05	98
3	$PBLL_{400}$	91.4	83.5	1.05	97

[a]PBLL was prepared through CE-catalyzed, biphasic polymerization of BLL-NCA. [b]Obtained from GPC, dn/dc = 0.059. [c]Obtained from NMR.

Table S2. Characterization of stimuli-responsive single-chain polypeptide nanoparticles before and after de-crosslinking.

Entry	Sample	D ($\times 10^{-11}$ m^2/ s)[a]	D_h (nm)[a]
1	DS_{200}	2.8	12.2
2	DS_{200} + DTT[b]	2.0	17.9
3	KT_{200}	2.6	13.3
4	KT_{200} + H^+ [c]	1.7	20.5

[a]Obtained from DOSY NMR. [b]De-crosslinking performed at 10 mM DTT. [c]De-crosslinking performed at aqueous pH = 4.5.

Supplementary References

1. Zhang, Y.; Wang, R.; Hua, Y.; Baumgartner, R.; Cheng, J. Trigger-responsive poly(β-amino ester) hydrogels. *ACS Macro Lett.* **2014,** *3*, 693-697.

2. Tian, Z.-Y.; Zhang, Z.; Wang, S.; Lu, H. A moisture-tolerant route to unprotected α/β-amino acid *N*-carboxyanhydrides and facile synthesis of hyperbranched polypeptides. *Nat. Commun.* **2021,** *12*, 5810.