ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Nanogel-Incorporated Physical and Chemical Hybrid Gels for Highly Effective Chemo–Protein Combination Therapy

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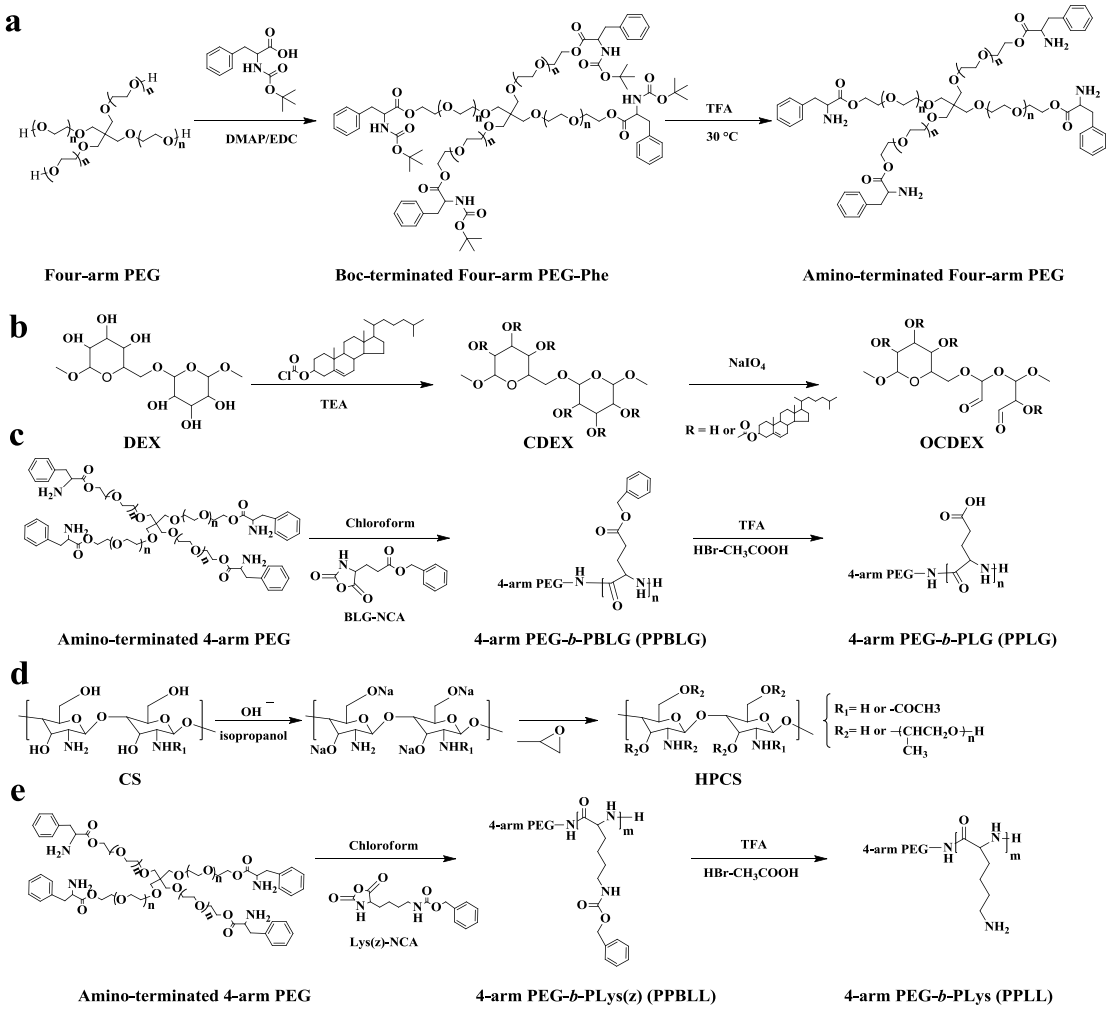
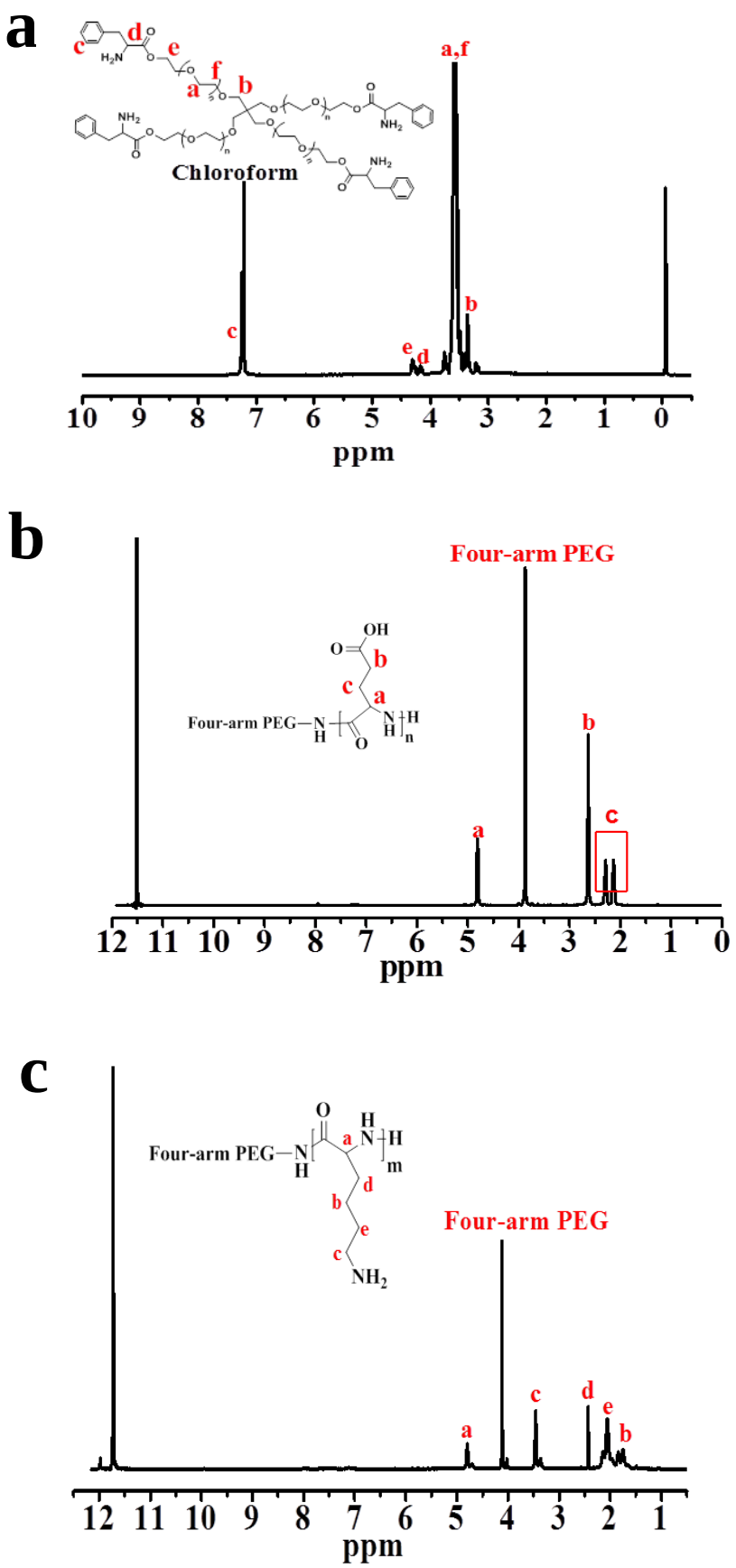
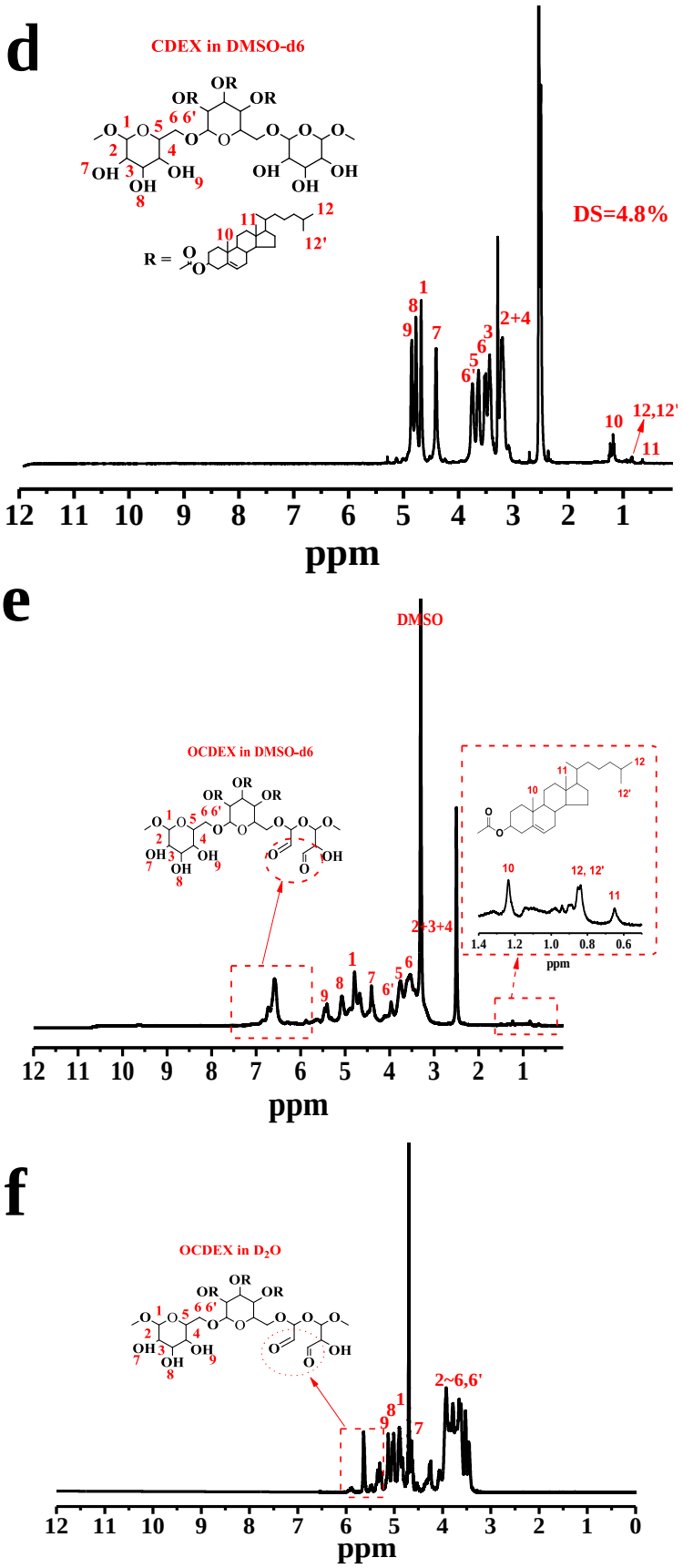
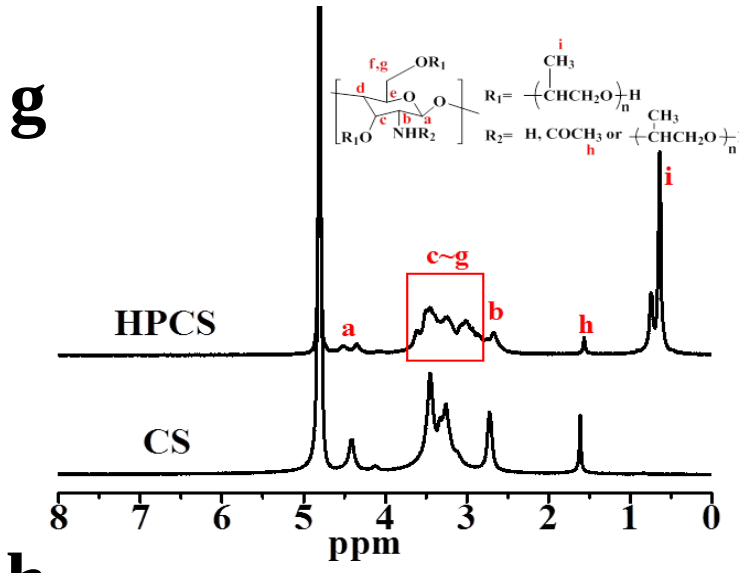
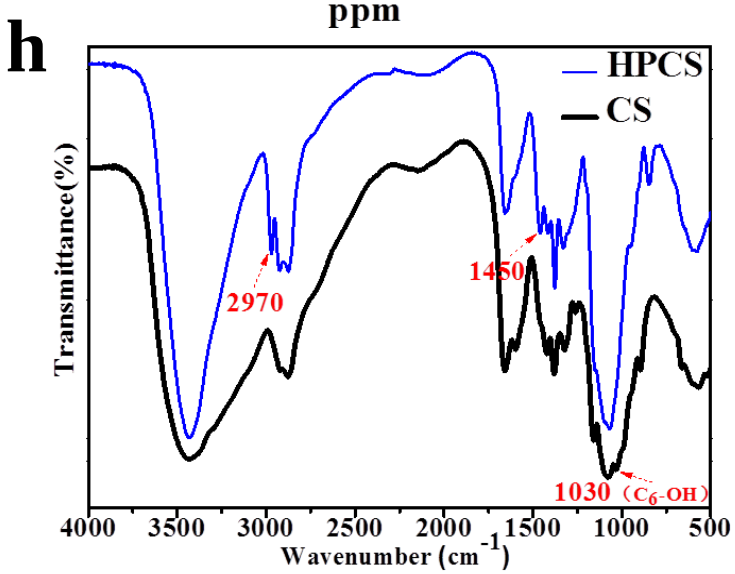


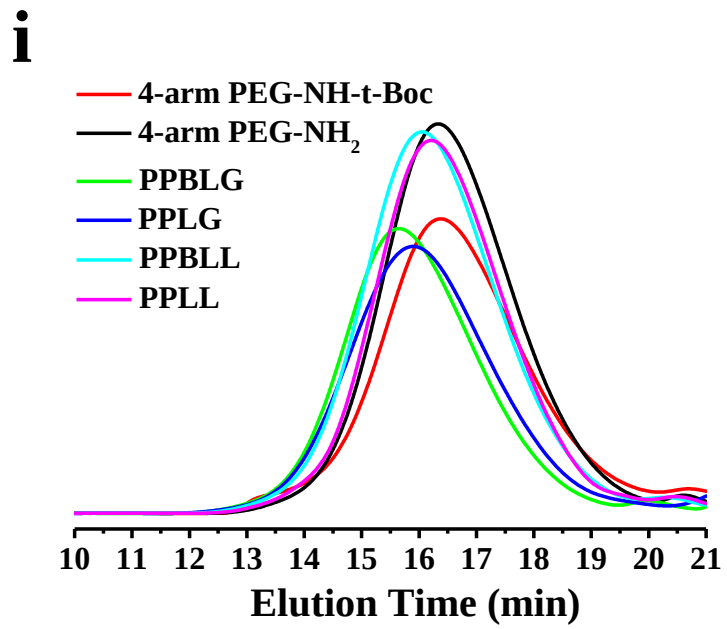
Figure S1. Synthetic routes of a) 4-arm amino-functionalized PEG (4-arm PEG-NH_2), b) OCDEX, c) PPLG, d) HPCS and e) PPLL.


a
Chloroform
a,f
b
c
e
d
10 9 8 7 6 5 4 3 2 1 0
ppm
b
Four-arm PEG
Four-arm PEG
OH
O
b
c
a
N
H
H
n
a
b
c
12 11 10 9 8 7 6 5 4 3 2 1 0
ppm
c
Four-arm PEG
O
a
H
N
H
m
d
b
e
c
NH2
Four-arm PEG
a
c
d
e
b
12 11 10 9 8 7 6 5 4 3 2 1
ppm


d
CDEX in DMSO-d6
DS=4.8%
ppm
e
DMSO
OCDEX in DMSO-d6
2~3+4
ppm
f
OCDEX in D2O
2~6,6'
ppm


g
HPCS
CS
a
b
c~g
h
i
8 7 6 5 4 3 2 1 0
ppm


h
HPCS
CS
Transmittance(%)
2970
1450
1030 (C6-OH)
4000 3500 3000 2500 2000 1500 1000 500
Wavenumber (cm-1)


i
4-arm PEG-NH-t-Boc
4-arm PEG-NH2
PPBLG
PPLG
PPBLL
PPLL
10 11 12 13 14 15 16 17 18 19 20 21
Elution Time (min)

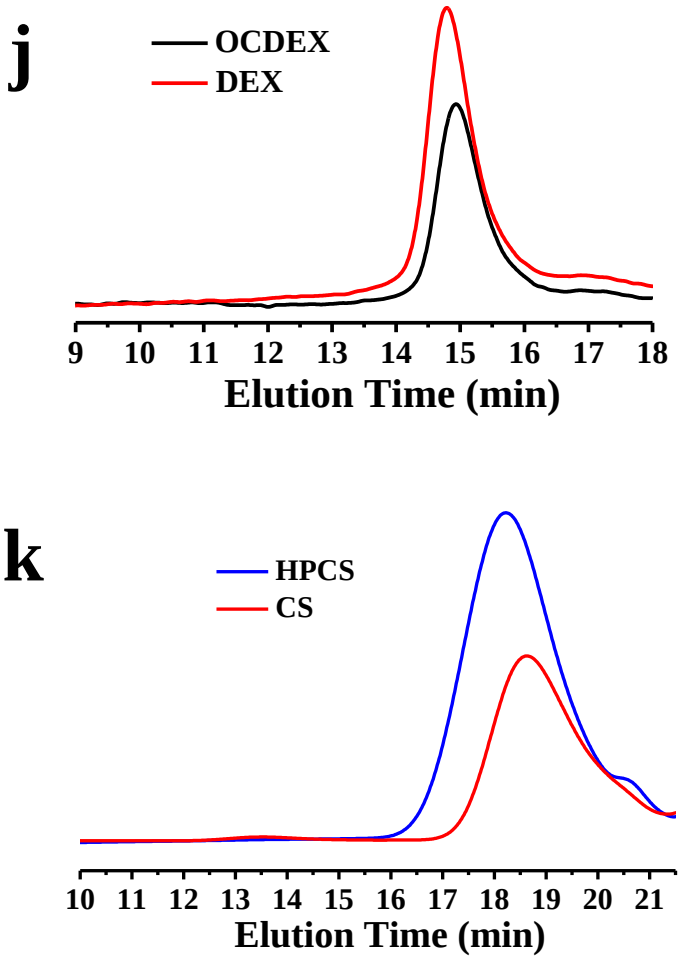


Figure S2. Structural characterization. ^{1}H NMR spectra of a) 4-arm PEG-NH_2 ($CDCl_3$), b) PPLG (CF_3COOD), c) PPLL (CF_3COOD), d) CDEX (DMSO-d_6), e) OCDEX (DMSO-d_6), f) OCDEX (D_2O), g) HPCS (D_2O) and CS (D_2O/CF_3COOH = 9/1). h) FTIR spectra of HPCS and CS. For b and c, the target degree of polymerization (DP) for a branch of each block was 150. The actual mean polymerization degree for a branch of PPLG was determined to be 132 by comparing integration areas of peak b (-CH_2CH_2O-, 4H, 4-arm PEG block) at 3.87 ppm with that of peak e (-CH_2COOH, 2H, PLG block) at 2.63 ppm. The integrated methylene protons signal (-CH_2CH_2O-, 4H) of 4-arm PEG block in the range of 4.18~4.02 ppm was compared to the integrated methylene protons signal (-CH_2NH_2, 2H) in side

groups of polypeptide in the range of 3.54~3.36 ppm, in order to calculate the mean DP for a branch of PPLL block, which was estimated to be 113. For d and e, by calculating the ratio integrated area for the protons signal at δ = 1.23 (-CH_3 at position 10 of cholesterol) and δ = 4.90 (anomeric protons at position 1 of the dextran skeleton), the estimated substitution degree (SD) of cholesterol is 4.2%. In the ^1H NMR spectroscopy of HPCS (g), the methyl protons in the hydroxypropyl moiety successively absorb at δ= 0.6~0.7. FTIR spectra (h) of CS and its derivatives show that the new peaks at 2970 cm^{-1} and 1450 cm^{-1} are associated with stretching vibration and bending vibration absorption peaks of methyl of the hydroxypropyl moiety. The absorption peak at 1030 cm^{-1} of the primary alcohol in CS, ascribed to C-O stretching vibration, completely disappears in HPCS, which demonstrates that the substitution occurs at C_6 position. Typical GPC curves of (i) 4-arm PEG-NH-t-Boc, 4-arm PEG-NH_2, PPBLG, PPLG, PPBLL, PPLL, (j) DEX, OCDEX and (k) CS, HPCS. All the GPC traces present a unimodal character indicating successful polymer synthesis.

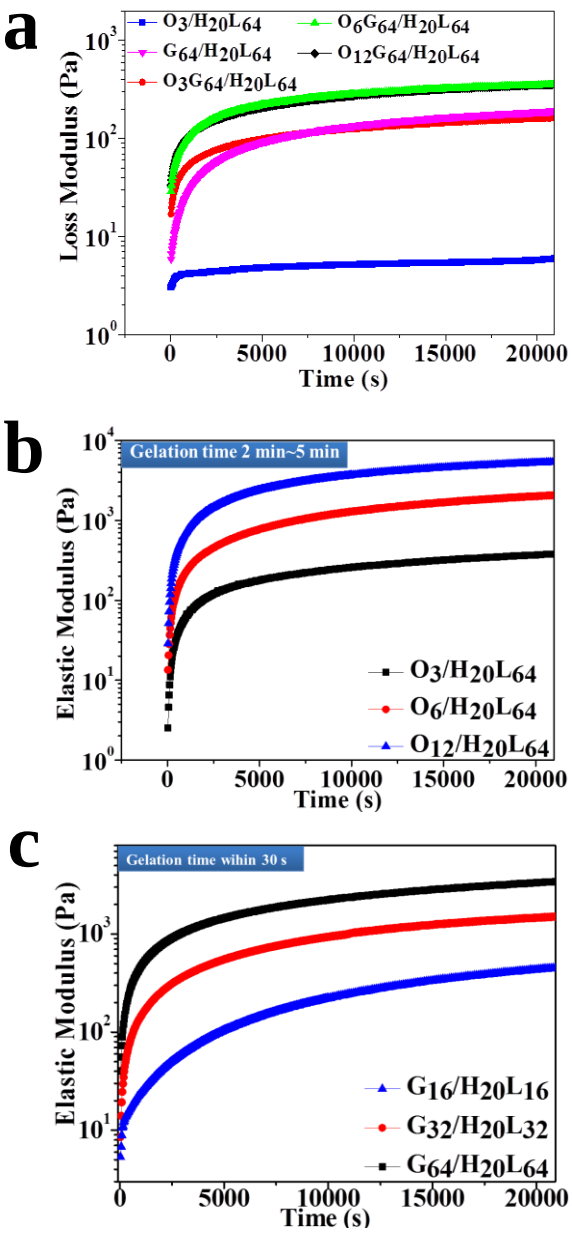


Figure S3. Oscillatory rheology of different *in situ* gel formulations. a) Loss modulus (G") as a function of time for double-crosslinking hydrogel formulations (O_xG_y/H_zL_w). Elastic modulus (G') versus time for single-crosslinking networks of b) the Schiff-base conjugating (O_x/H_zL_w) between aldehyde group-containing OCDEX nanogels and amino-bearing composites (HPCS/PPLL) and c) the ionic crosslinking (G_y/H_zL_w) of negatively charged PPLG with positively charged polysaccharide/polypeptide complex (HPCS/PPLL).

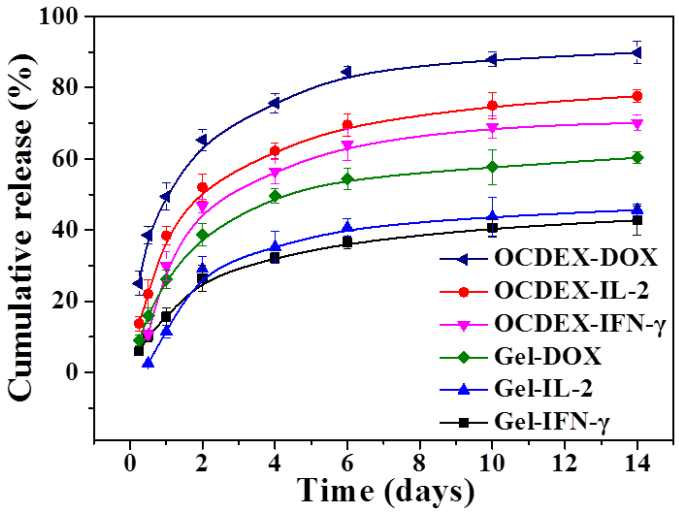


Figure S4. Cumulative release of IL-2, IFN-γ and DOX from nPCGs and OCDEX. Amphiphilic OCDEX nanogels was utilized as an outside criterion for comparison. The cumulative releases of IL-2, IFN-γ and DOX from OCDEX were 77.6%, 70.1% and 89.8%, respectively, at pH 7.4 after 14 days incubation, while a significantly slower release was observed from nPCGs (45.6%, 42.7% and 61.3%). Especially, DOX was released out at a relative faster rate probably due to its lower molecular weight. Results are shown as mean ± s.d. (n=3).

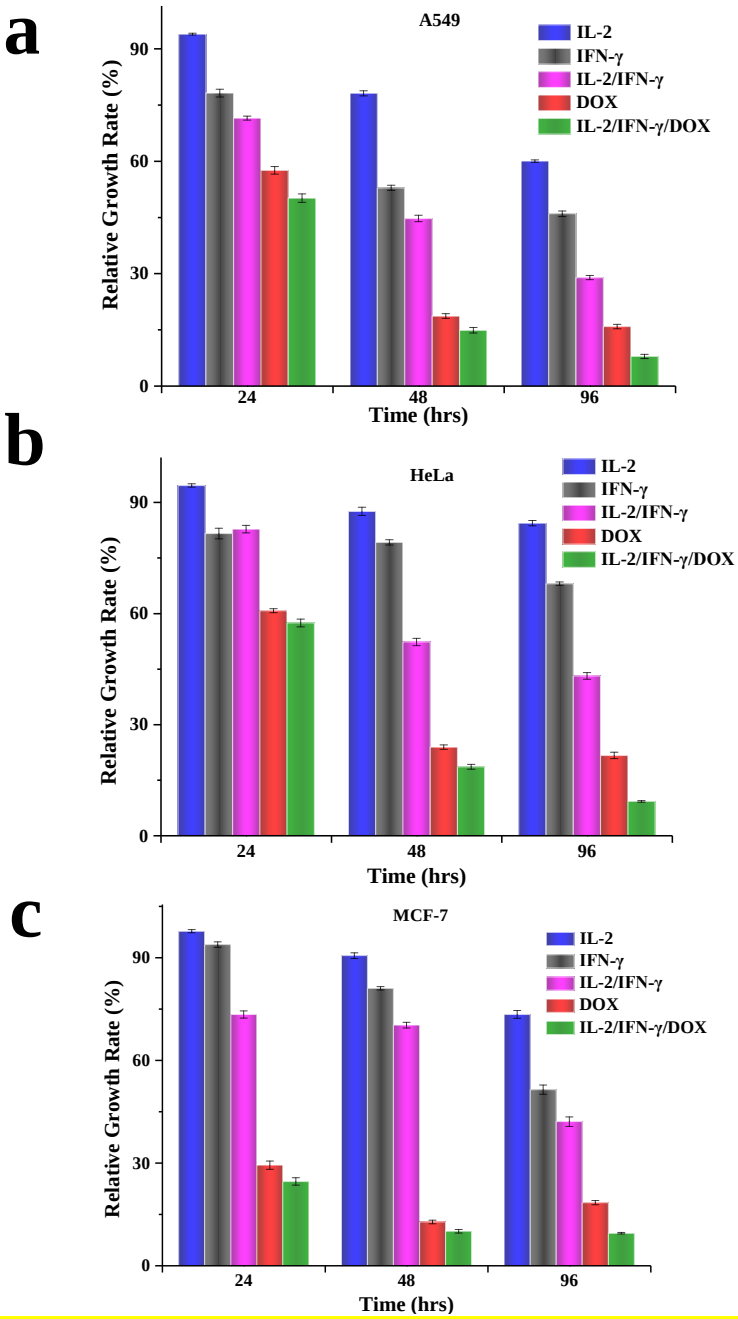


Figure S5. *In vitro* cytotoxicity of free drug solutions during different incubation time. a) A549. b) HeLa. c) MCF-7. After 96 h of incubation, the drug-loaded nPCGs revealed a slightly higher tumor cell viability as compared to the equal amounts of free agents determined from the release media described above.

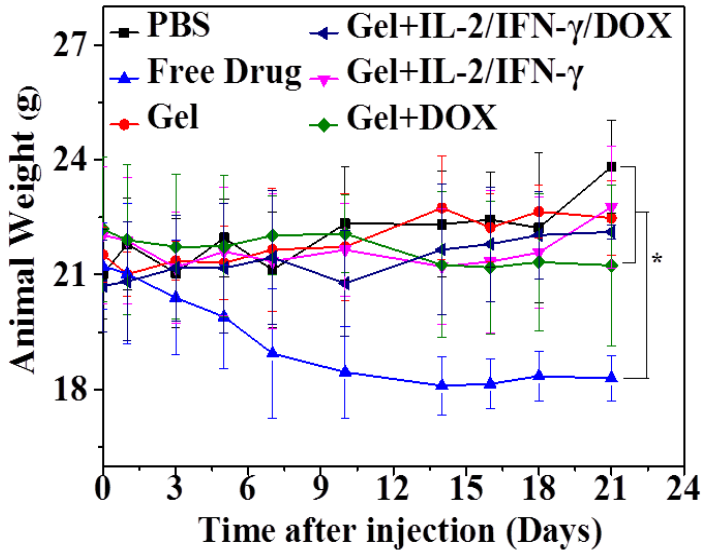


Figure S6. Body weight changes with the time of A549 inoculated BALB/c nude mice treated with different formulations. Data are shown as the average values ± s.d. (n=5). *: $P<0.05$.

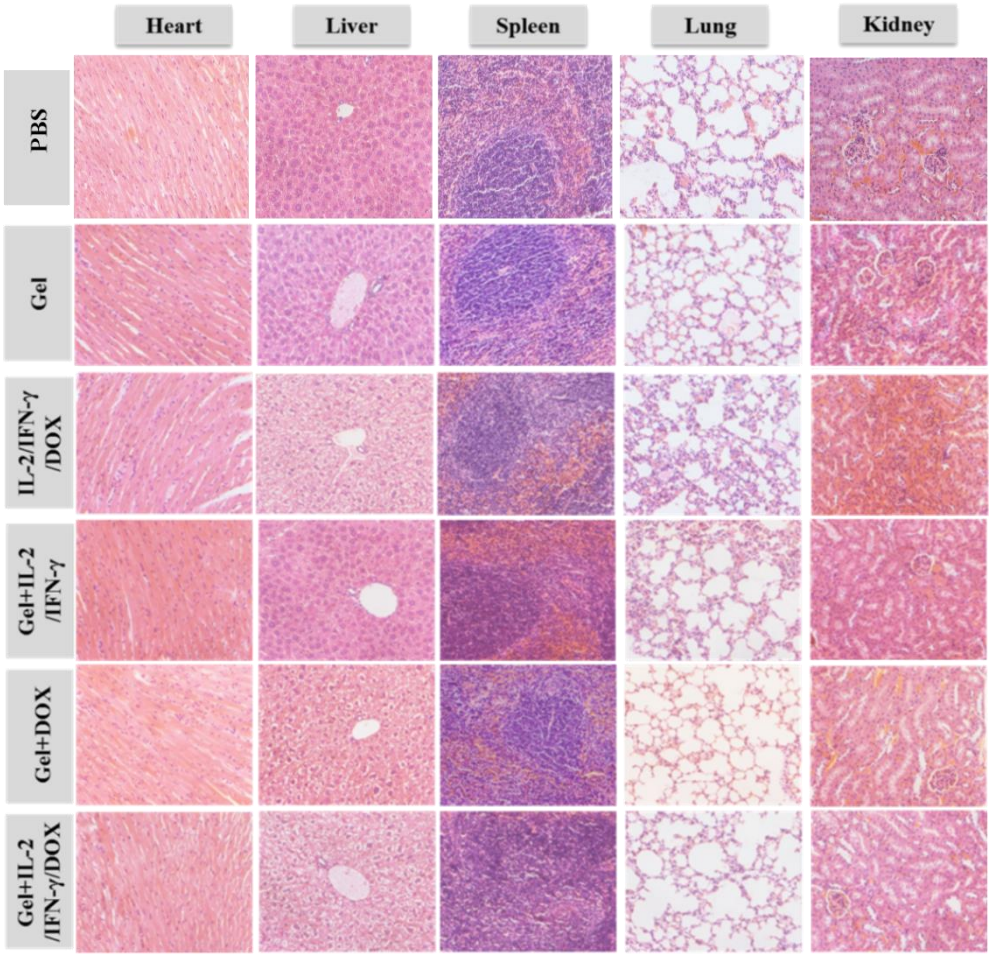


Figure S7. Histologic assessments of major organs with H&E staining in mice. To assess possible systemic toxicity, morphologic evaluation of H&E-stained sections of major organs including heart, liver, spleen, lung and kidney was performed for the mice treated with various formulations at day 21. The tumor-bearing mice treated with PBS and blank hydrogel groups displayed the same normal histological morphologies. Free IL-2/IFN-γ/DOX treated group exhibited notable kidney toxicity as well as myocardial damage due to a massive necrosis of heart muscle fibers with infiltration of inflammatory cells. In contrast, the treatment of the tumor-bearing mice with drug-loaded nPCGs groups displayed obviously reduced toxicity to kidney and heart, likely due to their sustained release manner.

Table S1. List of primers used for real-time PCR.

Gene	Genetic ID	Sense primer	Anti-sense primer
β-actin	BC002409	TGATGATATCGCCGCGCTC	ATCCTTCTGACCCATGCCCA
STAT1	NM_007315	TTGGCCCAGTGGATTGAGAG	GGGGCAGCGGTCATATGTTT
STAT5	L41142.1	ATCGAGGTCCGGCACTACTT	GGGCAGCGGTCATATGTTTTC
Caspase-9	NM_001229.3	TGTTCAGGCCCCATATGATCG	CTGGCCTGTGTCCTCTAAGC
Caspase-3	NM_004346.3	TCCTAGCGGATGGGTGCTAT	CTCACGGCCTGGGATTTCAA
BAX	NM_004324.3	AGCAGATCATGAAGACAGGG	TTCTTGGTGGACGCATCCTG
Bcl-2	NM_000633.2	CATGCGGCCTCTGTTTGATT	GTTGACTTCACTTGTGGCCC

Materials and Methods

The synthetic approach of 4-arm amino-functionalized PEG (4-arm PEG-NH_2). 4-arm PEG-NH_2 were synthesized by condensation reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl)/4-N, N-dimethylaminopyridine (DMAP) as the coupling reagents and then the deprotection of tert-butoxycarbonyl (t-Boc) group as depicted in Figure S1. Briefly, 4-arm Poly(ethylene glycol) (4-arm PEG) (mol wt ~ 10000, 10.0 g, 1.0 mmol), N-(tert-butoxycarbonyl)-L-phenylalanine (2.12 g, 8.0 mmol) and DMAP (0.98 mg, 8.0 mmol) were dissolved in dry DCM (50 mL) in a flame-dry flask, then EDC·HCl (7.68 g, 40.0 mmol) in DCM was added slowly to the solution with continuous stirring and the reaction was conducted at ambient temperature for 48 h. Then, the solution was further washed twice with dilute hydrochloric acid and alkaline. The obtained product was precipitated into excessive diethyl ether and dried under vacuum at room temperature for 24 h (Yield: 87%). Subsequently, 4-arm PEG-NH-t-Boc (8.0 g, 0.73 mmol) was dissolved in DCM (50 mL) at 25 °C in a flask. After trifluoroacetic acid (80 mL) was added, the solution was slowly stirred at 25 °C for 3 h and then the final product was precipitated into excessive diethyl ether and washed twice with diethyl ether. The precipitate was collected and dried under vacuum to a constant weight at room temperature (Yield: ~79%).

Synthesis of Four-Arm Star-Shaped Block Copolymer 4-arm Poly(ethylene glycol)-*b*-Poly(L-glutamic acid) (PPLG) and 4-arm Poly(ethylene

glycol)-*b*-Poly(L-lysine) (PPLL). γ-Benzyl-L-glutamate-N-carboxyanhydride (BLG-NCA) and ε-benzyloxycarbonyl-L-lysine-N-carboxyanhydride (BLL-NCA) were synthesized according to Daly's method.[1] For the synthesis, BLG-NCA (7.87 g, 29.9 mmol) or BLL-NCA (9.2 g, 30.1 mmol) was dissolved in dry chloroform (160 mL) in a flame-dry flask. The aforementioned dry 4-arm PEG-NH_2 (0.5 g, 0.05 mmol) was then added to the flask. The polymerization was performed at 30 °C for 3 d under a dry nitrogen atmosphere. Then, the solution was precipitated into excessive diethyl ether/ethanol (2/1, v/v) mixture. The obtained product was further washed twice with diethyl ether and dried under vacuum at room temperature for 24 h (Yields: both at 82.3~86.7%). Subsequently, four-arm poly(ethylene glycol)-*b*-poly(γ-benzyl-L-glutamate) (PPBLG) or four-arm poly(ethylene glycol)-*b*-poly[ε-(benzyloxycarbonyl)-L-lysine] (PPBLL) (3.0 g) was dissolved in trifluoroacetic acid (30 mL) at 0 °C in a flask. After HBr/acetic acid (33 wt%, 9.0 mL) was added, the solution was slowly stirred at 30 °C for 1 h and then the final product was precipitated into excessive chilled diethyl ether and puried by dialysis against deionized water for 3 days (MWCO 7000 Da). The obtained products, PPLL and PPLG star-shaped block copolymers, were collected as a white solid by lyophilization ($Yield_{PPLL}$: 76.5%, $M_{n,GPC}$ = 28400, M_w/M_n = 1.23; $Yield_{PPLG}$: 83.7%, $M_{n,GPC}$ = 32800, M_w/M_n = 1.31).

Synthesis of amphiphilic Oxidized Cholesteryl-bearing Dextran derivative (OCDEX) and Hydroxypropyl Chitosan (HPCS). Cholesteryl-bearing dextran

(CDEX) was obtained by esterification of dextran with cholesteryl chloroformate using anhydrous dimethyl sulfoxide (DMSO) and dichloromethane (DCM) as a mixed solution and triethylamine as a catalyst. Then the OCDEX was prepared according to the following process: $NaIO_4$ (2.46 g) was added to amphiphilic CDEX solution (1.86% w/v, 100 mL), shielded from light, with constant stirring at ambient temperature for 12 h. The OCDEX solution was dialyzed exhaustively (MWCO 3500) for 3 days against water, and pure OCDEX was obtained by lyophilization. The actual oxidation degree of OCDEX was determined to be ~49% by reaction with purpald (Aldrich).[2] The structures of CDEX and OCDEX were confirmed by ^1H NMR (DMSO-d_6, Bruker Avance-600 NMR spectrometer). HPCS was prepared from chitosan (CS) and propylene oxide according to the literature[3] with minor modifications. In brief, alkali chitosan (5.0 g) was mixed with isopropyl alcohol (50 ml) after being frozen and thawed. Then propylene oxide (65 mL) was added drop-wise and refluxed 6 h at 45 °C with continuous stirring. The resulting solution was neutralized by the addition of hydrochloric acid. Then the product was precipitated twice from ethanol and filted to give the yellowish hydroxypropyl chitosan (HPCS) derivatives. The chemical structures of HPCS and CS were characterized using a ^1H NMR spectrometer (Bruker Avance-600 NMR spectrometer, Billerica, MA). D_2O/CF_3COOH (9/1) (for CS) or D2O (for HPCS) was used as solvents. Fourier transform infrared spectroscopy (FTIR) were measured with a Bruker VERTEX 70 spectrometer by the KBr pellet method.

References

[1] T. J. Deming, *Prog. Polym. Sci.* **2007**, *32*, 858.
[2] M. Quesenberry, Y. Lee, *Anal. Biochem.* **1996**, *234*, 50.
[3] S. S. Kim, S. J. Kim, Y. D. Moon, Y. M. Lee, *Polymer* **1994**, *35*, 3212.