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Intercalation-mediated Nucleic Acid Nanoparticles for siRNA Delivery

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Kejin Zhou, Petra Kos, Yunfeng Yan, Hu Xiong, Yi-Li Min, Karina A. Kinghorn, Jonathan T. Minnig, Jason B. Miller, and Daniel J. Siegwart*

*The University of Texas Southwestern Medical Center, Simmons Comprehensive Cancer Center, Department of Biochemistry, Dallas, Texas 75390 (USA) *E-mail: daniel.siegwart@utsouthwestern.edu*

Electronic Supplementary Information (ESI)

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1. Materials and Instrumentation

1.1 Materials

1,4-Diaminobutane, cystamine dihydrochloride, methyl acrylate, ethylenediamine, 1-pyrenebutyric acid, 1-naphthaleneacetic acid, dodecanoic acid, cholesteryl chloroformate, and all other unspecified chemicals were purchased from Sigma-Aldrich. Dansyl chloride was purchased from Acros Organics. Dulbecco's Modified Eagle Media (DMEM), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. Cell Mask Orange was purchased from Molecular Probes. ONE-Glo + Tox was purchased from Promega. All organic solvents were purchased from Fisher Scientific or Sigma-Aldrich and purified with a solvent purification system (Innovative Technology). siLuc siRNA targeting luciferase (siLuc; sense strand: 5'-GAUUAUGUCCGGUUAUGUA[dT][dT]-3'; anti-sense strand: 3'-UACAUAACCGGACAUAAUC[dT][dT]-5'), siRNA targeting ubiquitin B (siUBB; sense strand: 5'- GUAUGCAGAUCUUCGUGAA [dT][dT]-3'; anti-sense strand: 3'-CATACGTCTAGAGCACUACT[dT][dT]-5'), siRNA targeting FVII (siFVII; sense: 5'-GGAUCAUCUCAAGUCUUAC[dT][dT]-3'; ANTISENSE: 3'-GUAAGACUUGAGAUGAUCC[dT][dT]-5'), and siRNA used as a control sequence (siCtrl; sense: 5'-GCGCGAUAGCGCGAAUAUA[dT][dT]-3'; antisense: 3'-UAUAUUCGCGCUAUCGCGC[dT][dT]-5') were custom synthesized by Sigma-Aldrich. Cy5.5-siLuc had the same sequence as siLuc, but was labeled with the Cy5.5 dye at the end terminus. The PEG2000-C16 lipid used in this study was synthesized as reported in our previous study.[1] The molecular weight of this PEG-lipid is 2980 g/mol.

1.2 Instrumentation

a.) Nuclear Magnetic Resonance (NMR) spectroscopy. ^{1}H and ^{13}C NMR were performed on a Varian 500 MHz spectrometer.

b.) Mass spectroscopy (MS). MALDI-TOF MS was performed on a Voyager-DE PRO biospectrometry workstation (Applied Biosystems) using 2,5-dihydroxy benzoic acid as the matrix.

c.) Flash chromatography. Flash chromatography was performed on a Teledyne Isco CombiFlash Rf-200i chromatography system equipped with UV-vis and evaporative light scattering detectors (ELSD).

d.) Transmission Electron Microscopy (TEM) was performed on a FEI Tecnai G2 Spirit Biotwin at an accelerated voltage of 120 kW. For sample preparation, a drop of formulated NP was placed on a carbon film covered TEM grid, excess liquid was then wicked by filter paper and stained with the solution of 3% uranyl acetate. The copper grid was then dried.

e.) Analysis of NP size and zeta potential. Particle sizes and zeta potential were measured by Dynamic Light Scattering (DLS) using a Malvern Zetasizer Nano ZS (He-Ne laser, λ = 632 nm).

f.) Studies of siRNA interaction with each CC/SS-IG inside nanoparticles. CD spectra were measured by JASCO J-815 Circular Dichroism Spectropolarimeter.

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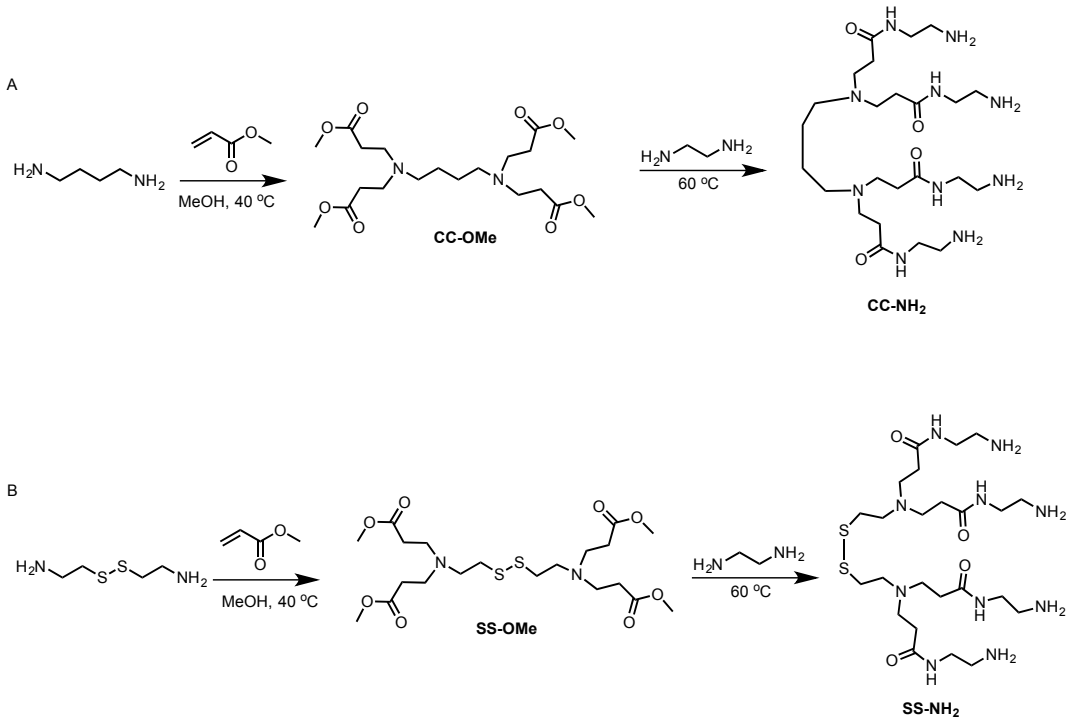
f.) Analysis of cellular uptake of the CC/SS-IG nanoparticles. The cellular uptake of the CC/SS-IG nanoparticles was analyzed using a Zeiss LSM-710 confocal microscope and a BD LSR Fortessa flow cytometry cell analyzer.

2. Chemical syntheses and analysis

2.1 CC-NH_2 and SS-NH_2 syntheses

1,4-Diaminobutane (2.5 g, 28.4 mmol) was added into a 100 mL round-bottom flask with 50 mL anhydrous methanol. Then, methyl acrylate (26 mL, 284 mmol) containing 100 ppm hydroquinone radical inhibitor was added. The reaction solution was heated to 40 °C for 72 hours. After the reaction was complete, methanol and excess methyl acrylate was removed to yield a viscous light yellow liquid. The product **CC-OMe** (9.1 g, 74%) was purified by flash chromatography with gradient eluent of hexane and ethyl acetate containing 2% triethylamine. The synthesis of **SS-OMe** (89%) was similar to that of **CC-OMe**. Their structures were verified by NMR (**Figure S1-S2**) and mass spectroscopy.

CC-OMe (5.0 g, 11.6 mmol) and ethylenediamine (15.1 mL, 2.3 mol) were added into a 100 mL round-bottom flask. The reaction was heated to 50 °C and continued until completion, as monitored by checking the conversion yield of ester into amide bond with NMR. Excess ethylenediamine and methanol were removed by rotary evaporation to yield a viscous light yellow liquid. The viscous liquid **CC-NH_2** was dried under high vacuum for the next reactions and used without further purification. The synthesis of **SS-NH_2** was similar to that of **CC-NH_2**.



Scheme S1. Synthetic scheme of **CC-NH_2** and **SS-NH_2**.

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2.2 CC-IG and SS-IG syntheses

General procedure for synthesis of CC-NA, SS-NA, CC-PY, SS-PY, CC-DD, and SS-DD

Synthesis of **CC-NA** is described as an example. In a 20 mL reaction vial, 1-naphthaleneacetic acid (409 mg, 2.20 mmol), HOBt (409 mg, 2.20 mmol) and DIEA (0.26 mL, 1.47 mmol) were dissolved in 8 mL dry DMF. Then, DIC (0.51 mL, 3.30 mmol) was added to the above solution and stirred at room temperature for 30 min. After addition of **CC-NH_2** (200 mg, 0.37 mmol) in 2 mL dry DMF, the reaction was stirred at room temperature for 24 addition hours. At that point, the product was visible in the mass spectrum. After the reaction was stopped, the reaction solution was dialyzed in methanol with 1 kD-cut-off dialysis tubing. Then, the dialysis solution was collected, concentrated and purified by flash chromatography to yield the **CC-NA** product. All structures were verified by NMR (**Figure S3-S8**) and mass spectroscopy.

General procedure for synthesis of CC-DA, SS-DA, CC-CH, and SS-CH

Synthesis of **CC-DA** is described as an example. In a 100 mL dried round-bottom flask, **CC-NH_2** (200 mg, 0.38 mmol) and DIEA (0.38 mL, 2.20 mmol) were dissolved in 30 mL dry chloroform. Then, dansyl chloride (587 mg, 2.20 mmol) in 20 mL dry chloroform was added dropwise into the above solution in an ice water bath. After addition of dansyl chloride, the reaction solution was stirred at room temperature for an additional 24 hours, when the product was shown in mass spectrum. Chloroform was removed and 200 mL DCM was added. The organic phase was washed with 50 mL brine water three times and dried with anhydrous $MgSO_4$. The dried solution was concentrated and purified by flash chromatography to yield the pure **CC-DA** product. All structures were verified by NMR and mass spectroscopy (**Figure S9-S12**).

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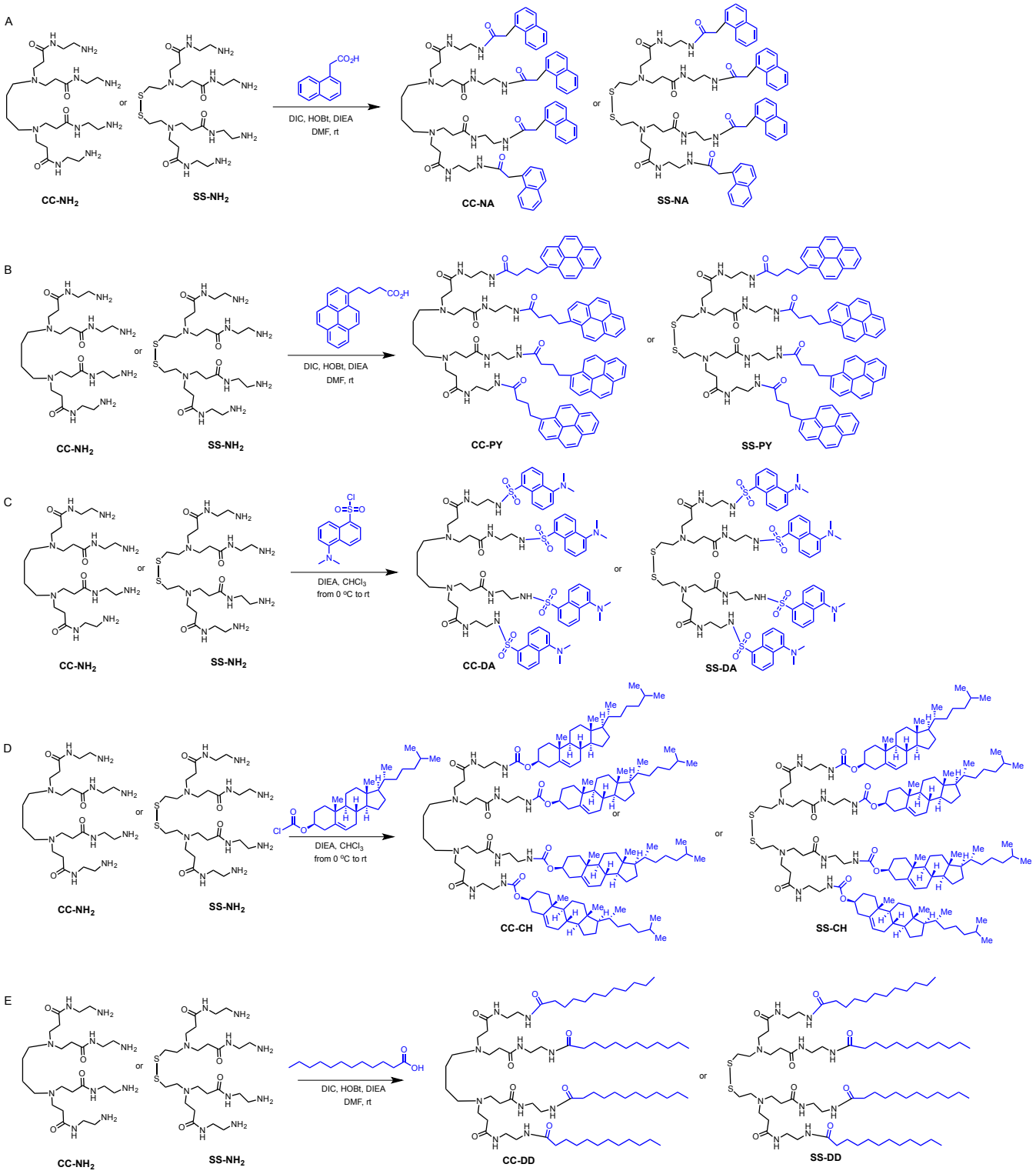

Scheme S2. Synthetic scheme of **CC-IGs** and **SS-IGs**.

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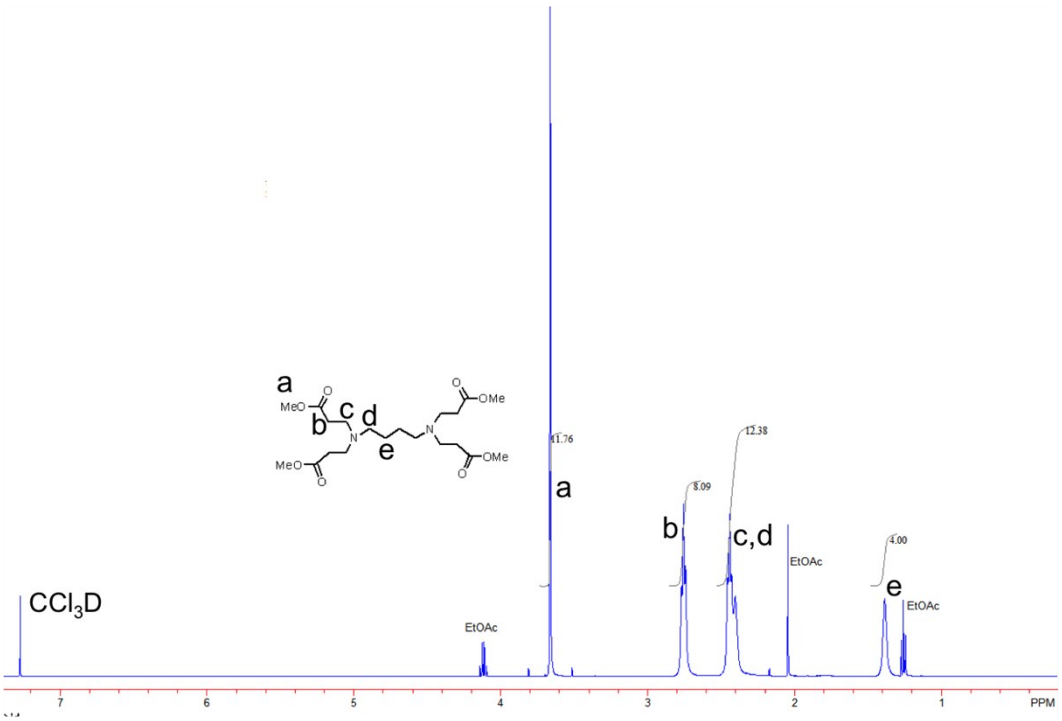

Figure S1. ^{1}H NMR of **CC-OMe** in chloroform-d.

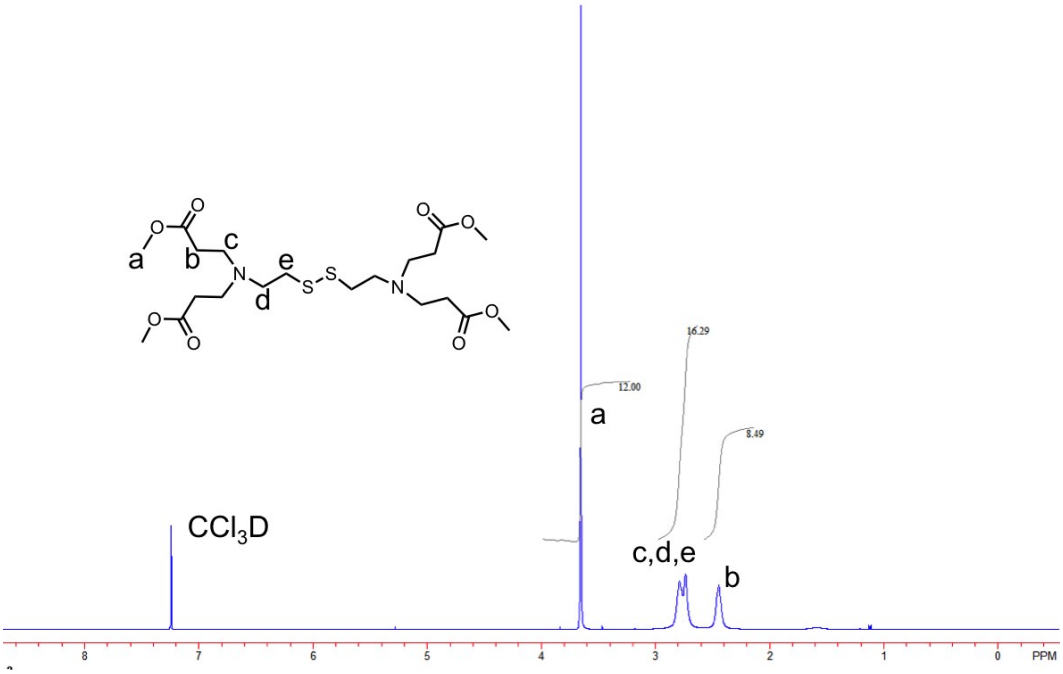

Figure S2. ^{1}H NMR of **SS-OMe** in chloroform-d.

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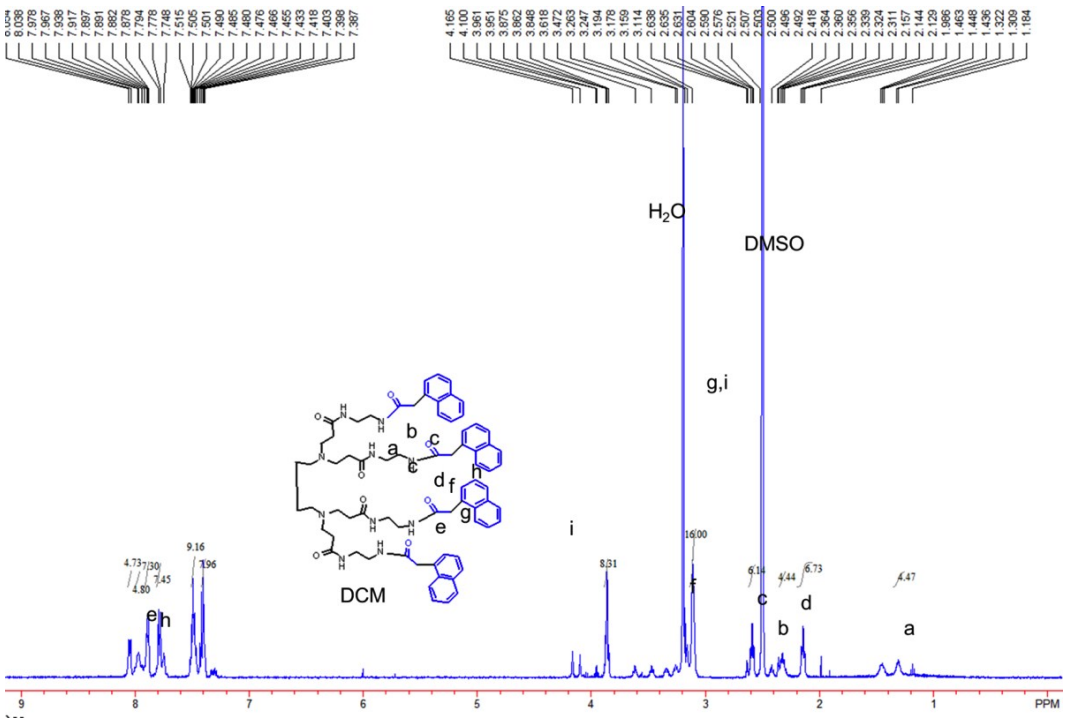

Figure S3. ^{1}H NMR of **CC-NA** in DMSO-d6. MS (MALDI-TOF, m/z) calc. for $C_{72}H_{84}N_{10}O_8$: 1216.65, found: 1216.43.

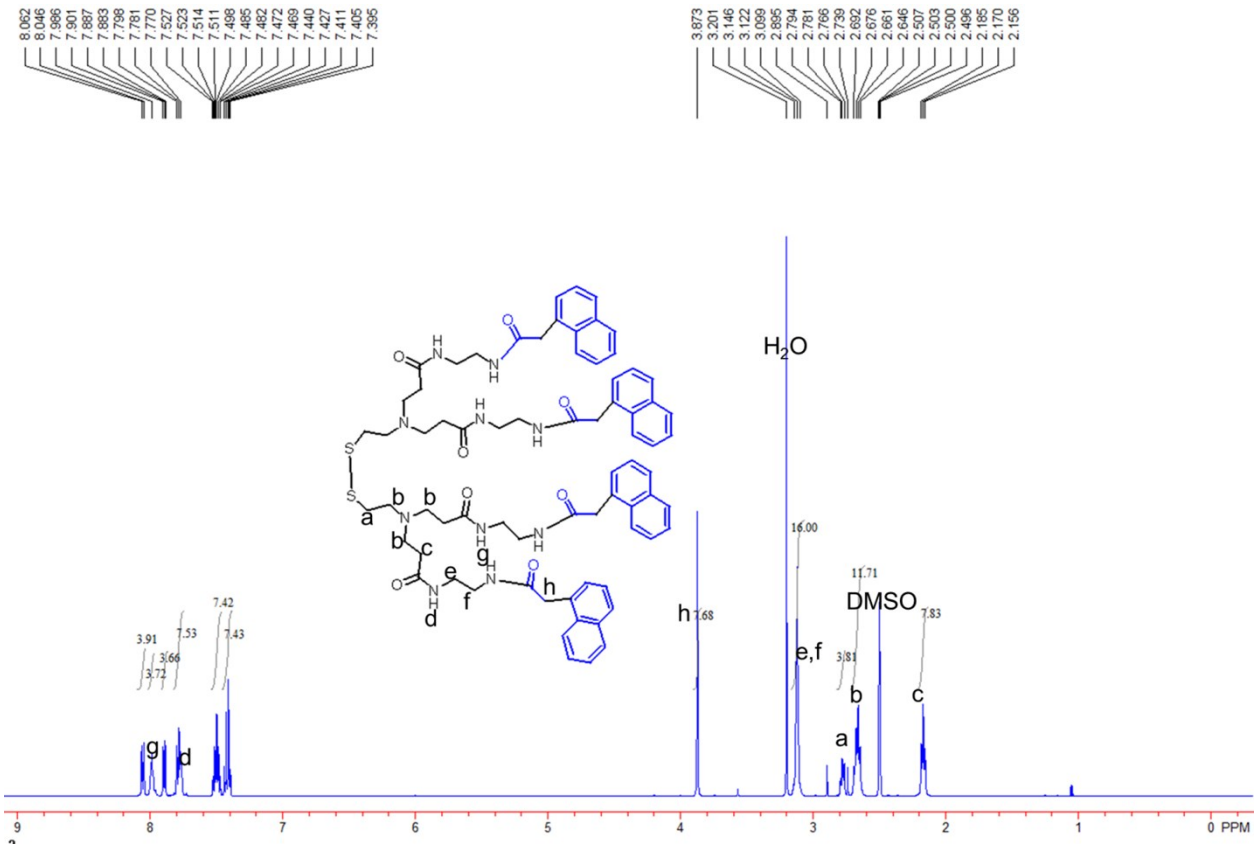

Figure S4. ^{1}H NMR of **SS-NA** in DMSO-d6. MS (MALDI-TOF, m/z) calc. for $C_{72}H_{84}N_{10}O_8S_2$: 1280.59, found: 1280.79.

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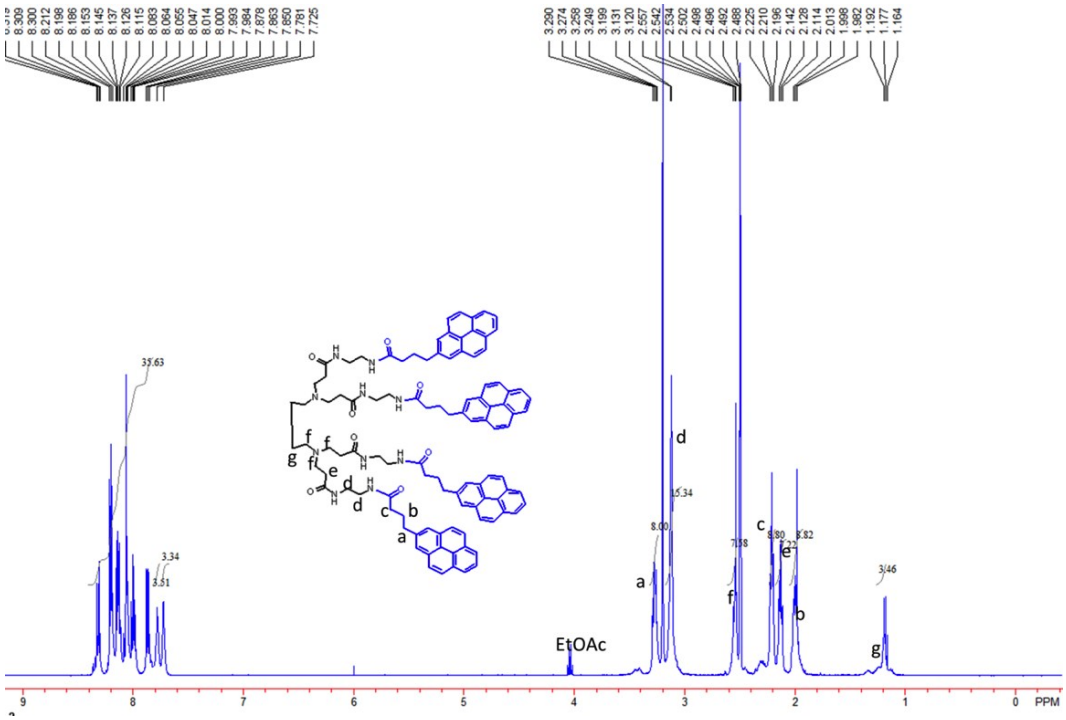

Figure S5. ^{1}H NMR of **CC-PY** in DMSO-d6. MS (MALDI-TOF, m/z) calc. for $C_{104}H_{108}N_{10}O_8$: 1624.84, found: 1624.67.

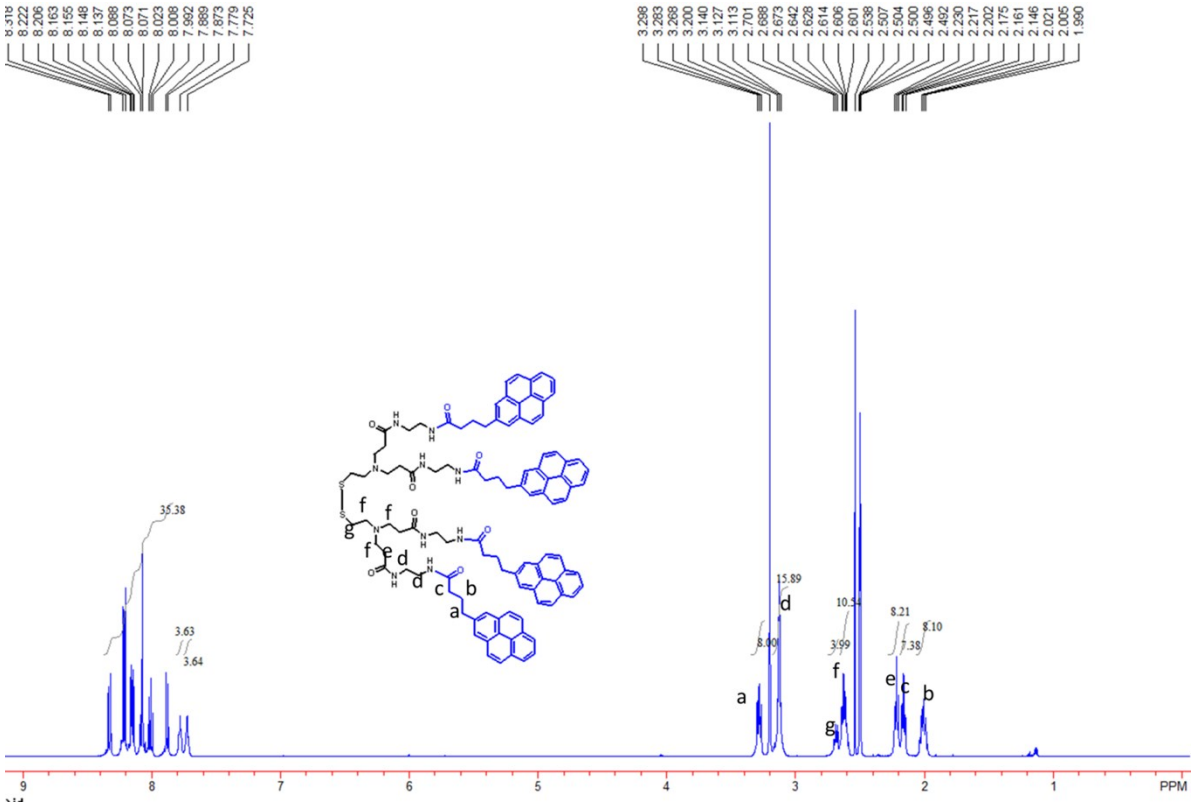

Figure S6. ^{1}H NMR of **SS-PY** in DMSO-d6. MS (MALDI-TOF, m/z) calc. for $C_{104}H_{108}N_{10}O_8S_2$: 1688.78, found: 1689.10.

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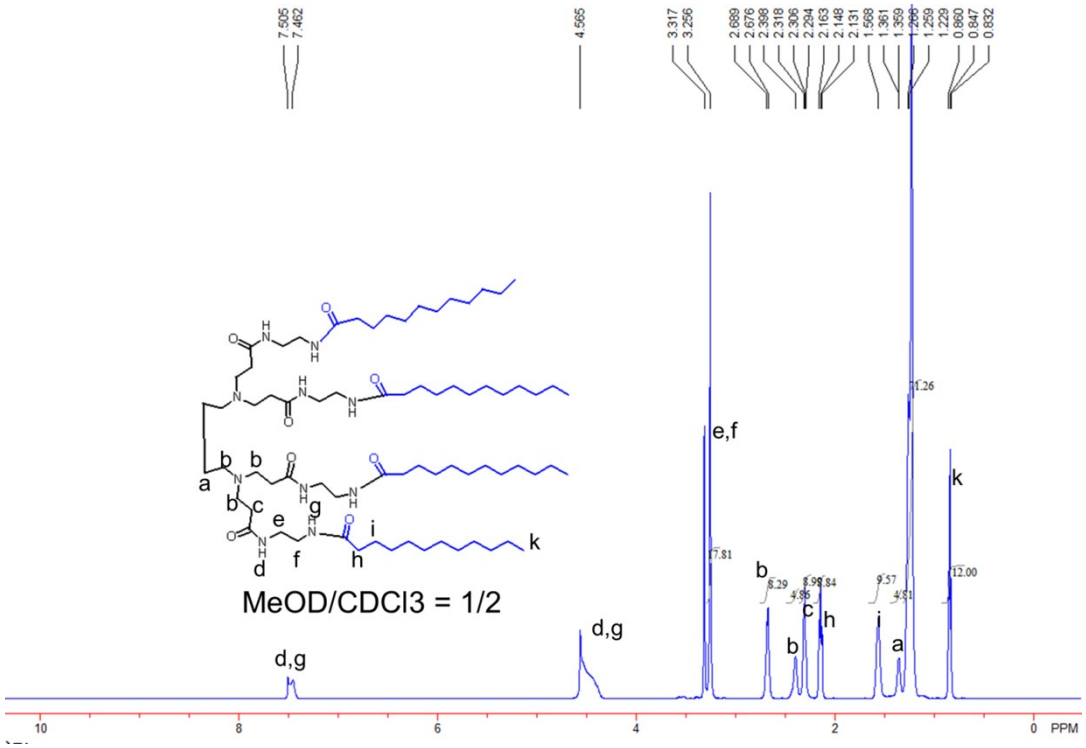

Figure S7. ^{1}H NMR of **CC-DD** in the mixed solvent of MeOD and $CDCl_3$. MS (MALDI-TOF, m/z) calc. for $C_{72}H_{140}N_{10}O_8$: 1273.09, found: 1272.86.

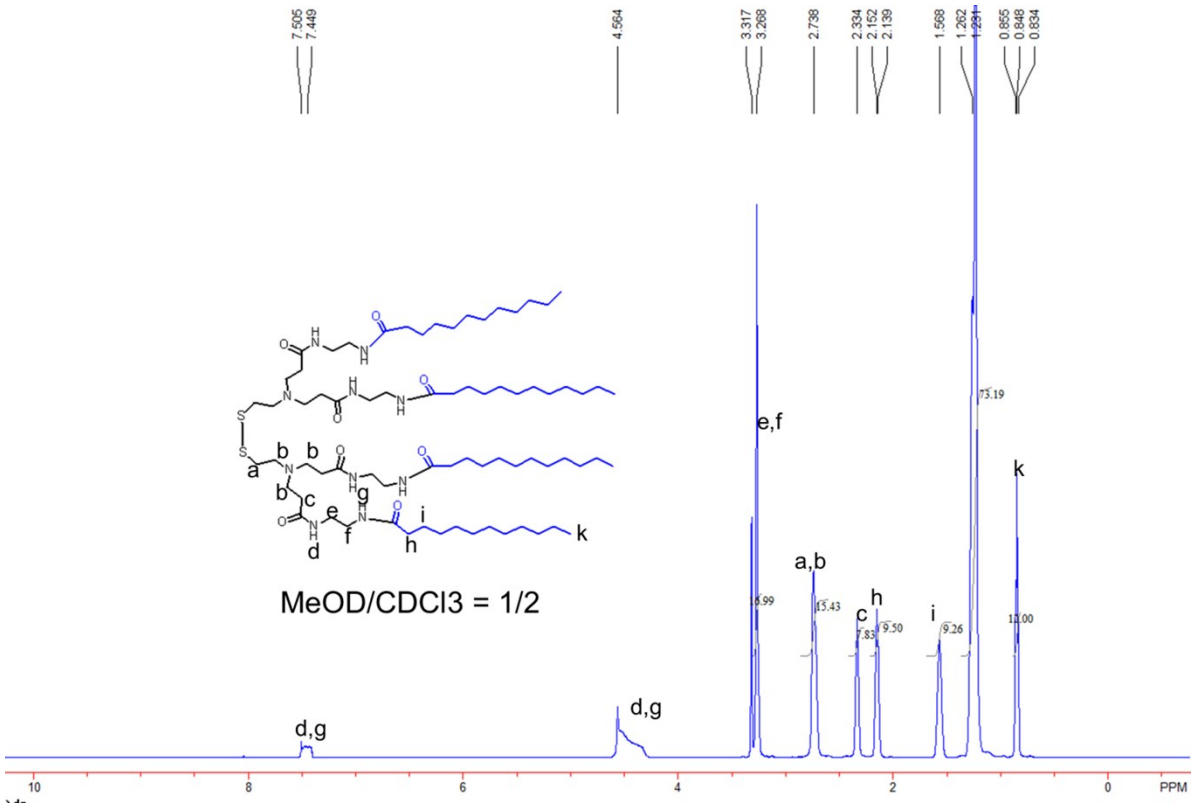

Figure S8. ^{1}H NMR of **SS-DD** in the mixed solvent of MeOD and $CDCl_3$. MS (MALDI-TOF, m/z) calc. for $C_{72}H_{140}N_{10}O_8S_2$: 1337.03, found: 1336.45.

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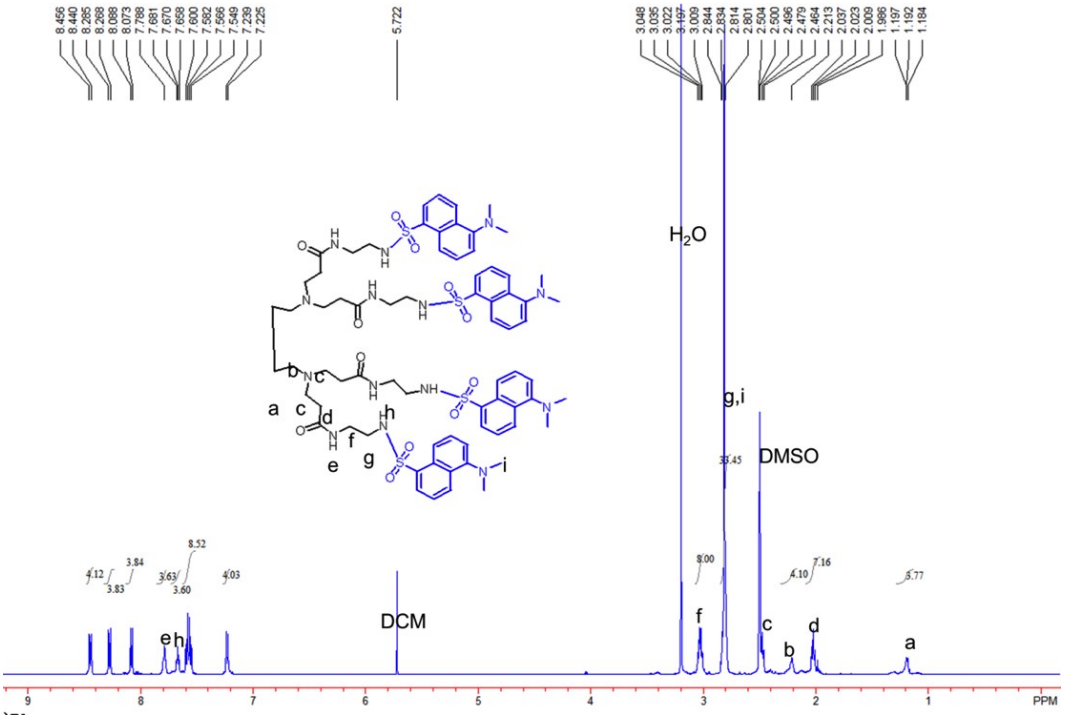

Figure S9. ^{1}H NMR of **CC-DA** in DMSO-d6. MS (MALDI-TOF, m/z) calc. for $C_{72}H_{96}N_{14}O_{12}S_4$: 1476.62, found: 1476.41.

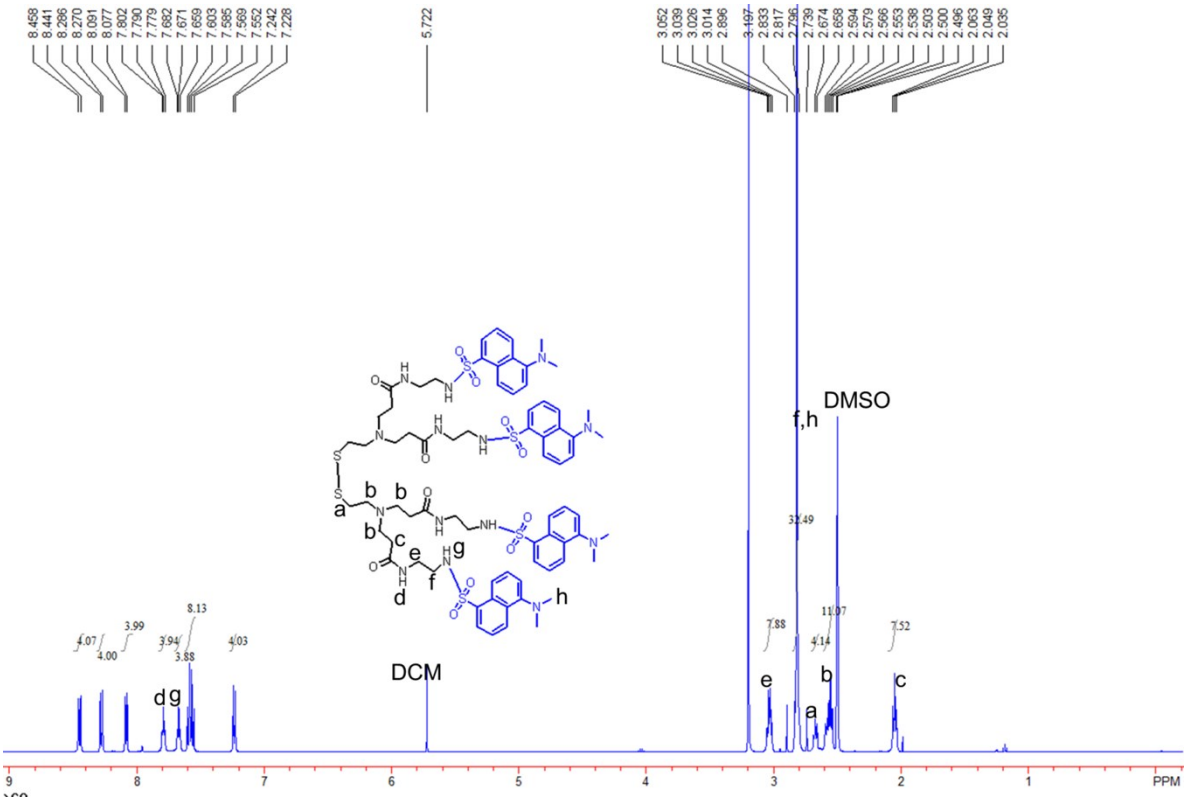

Figure S10. ^{1}H NMR of **SS-DA** in DMSO-d6. MS (MALDI-TOF, m/z) calc. for $C_{72}H_{96}N_{14}O_{12}S_6$: 1540.57, found: 1541.43.

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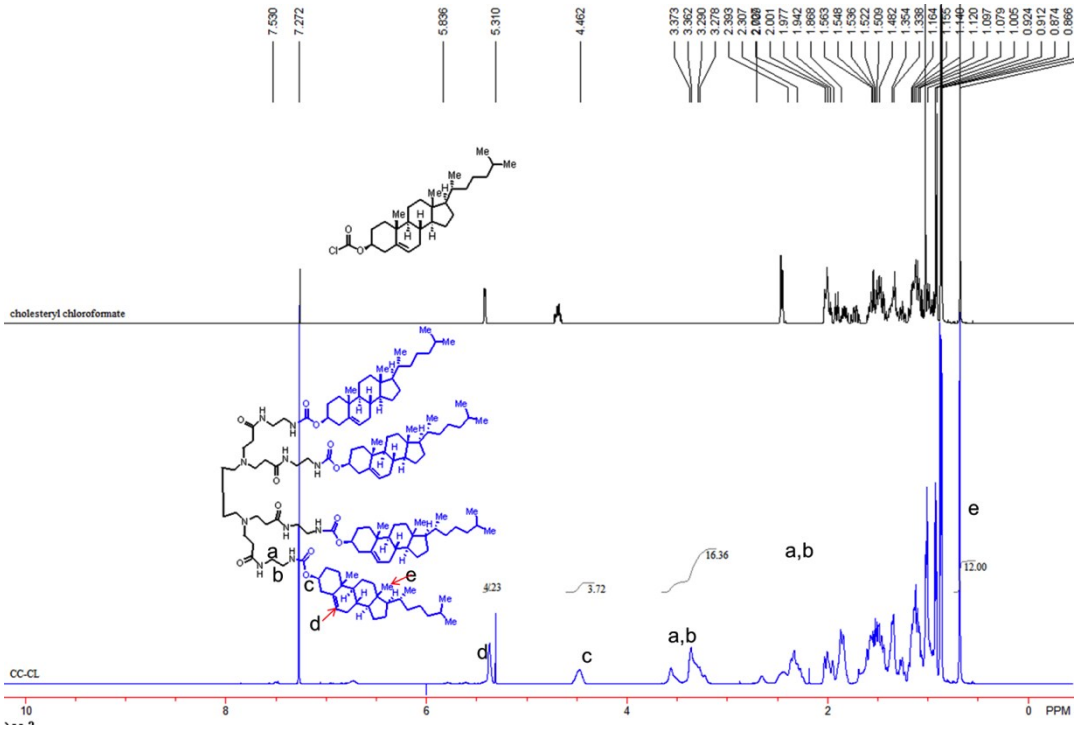

Figure S11. ^{1}H NMR of **CC-CH** in chloroform-d. MS (MALDI-TOF, m/z) calc. for $C_{136}H_{228}N_{10}O_{12}$: 2193.75, found: 2193.82.

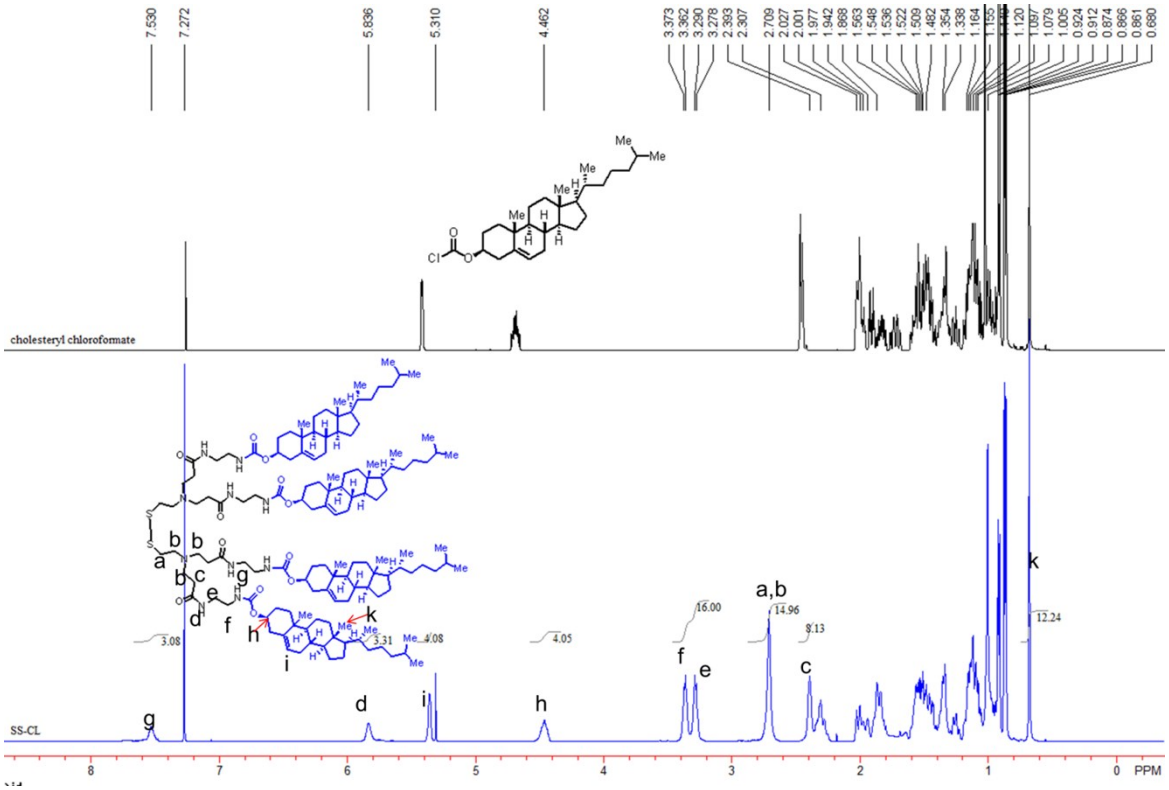

Figure S12. ^{1}H NMR of **SS-CH** in chloroform-d. MS (MALDI-TOF, m/z) calc. for $C_{136}H_{228}N_{10}O_{12}S_2$: 2257.70, found: 2257.93.

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3. Formulation and biological studies of CC/SS-IG nanoparticles

3.1 Formulation of CC/SS-IG nanoparticles

Optimization of nanoparticle formulation using CC-DA

The molar ratio of NP stabilizing PEG-lipid was optimized to formulate stable **CC-DA** nanoparticles. To make the mixed solution of **CC-DA** with PEG-lipid at a series of molar ratios of CC-DA/PEG-lipid, **CC-DA** was dissolved to make its final concentration 1.52 mM in DMSO solution containing PEG-lipid at a series of concentrations: 0.052 nM (molar ratio of **CC-DA**/PEG-lipid: 30), 0.104 mM (15), 0.220 mM (7), 0.384 mM (4), 0.616 mM (2.5) and 1.024 mM (1.5).

Under sonication, 150 µL of the above DMSO solution of **CC-DA**/PEG-lipid was added dropwise into 450 uL siRNA solution (5.13 µM, 0.067 mg/mL) in 10 mM citrate buffer (pH =3.0) (citric acid / sodium citrate). The yielded solution was dialyzed in 10 mM citrate buffer (pH = 3.0) for 12 hours and then in sterile PBS buffer for another 12 hours. The dialyzed solution was collected and diluted to 1.0 mL of total volume with addition of sterile PBS. The final **CC-DA** nanoparticle solutions with siRNA (2.31 µM, 0.040 mg/mL) were used to characterize the size and polydispersity using a Malvern Zetasizer Nano ZS up to two weeks. After one week, **CC-DA** nanoparticle solution was characterized with TEM.

Formulation of CC/SS-IG nanoparticles for other studies

The **CC/SS-IG** nanoparticles were formulated with molar ratios of **CC/SS-IG** to PEG lipid at 2.5 on the basis of the optimization of **CC-DA** formulated nanoparticles. Each **CC/SS-IG** was dissolved to make its final concentration 1.52 mM in DMSO solution containing PEG-lipid at the concentration of 0.616 mM. Under sonication, 150 µL of the above DMSO solution was added dropwise into 450 uL siRNA (siLuc, siUBB, siFVII or siCtrl) solution (5.13 µM, 0.067 mg/mL) in 10 mM citrate buffer (pH =3.0). The yielded solution was dialyzed in 10 mM citrate buffer (pH = 3.0) for 12 hours and then in sterile PBS buffer for another 12 hours. The dialyzed solution was collected and diluted to 1.0 mL of total volume with addition of sterile PBS, yielding the final **CC/SS-IG** nanoparticle solution with siRNA (2.31 µM, 0.030 mg/mL) for other studies.

3.2 Determination of apparent pKa of formulated CC/SS-IG nanoparticles

Following reported procedures,[2, 3] the TNS assay was used to determine the pK_a of each **CC/SS-IG** formulated nanoparticles comprised of **CC/SS-IG** and PEG-lipid at the mole ratio of 2.5 in PBS. The nanoparticle solutions were formulated with the same method without siRNA. 6-(*p*-Toluidino)-2-naphthalenesulfonic acid sodium salt (TNS) was prepared as a 20 µM stock solution in milliQ water. The formulated nanoparticles were diluted to 250 µM total lipid in 100 µL volume per well in 96-well plates with a series of 10 mM HEPES / 10 mM MES / 10 mM ammonium acetate / 130 mM NaCl buffer solutions, where the pH ranged from 2.5 to 8.5. The certain volume of TNS stock solution was added into each well to give a final concentration of 2 µM. The plate was measured using an InfiniTe F/M200 Pro microplate reader (Tecan) with excitation and emission wavelengths of 321 nm and 445 nm. Four replicates were used for each data point. For **CC-PY**, **SS-PY**, **CC-DA** and **SS-DA**, nanoparticle solutions without addition of TNS were used as blank controls. A sigmoidal fit analysis was applied to the fluorescence data and the pKa was measured as the pH value where the fluorescence intensity is half-maximal (**Table S1**).

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3.3 Evaluation of siRNA binding at different conditions

The Quant-iT RiboGreen RNA assay (Invitrogen) was used to evaluate the siRNA binding with each **CC/SS-IG** in pH 7.4 PBS buffer and pH 3.0 citrate buffer, respectively. Each **CC/SS-IG** was dissolved to make its final concentration 1.52 mM in DMSO solution containing PEG-lipid at the concentration of 0.616 mM. Under sonication, 150 µL of the above DMSO solution was added dropwise into 450 uL siRNA solution (5.13 µM, 0.067 mg/mL) in pH 7.4 PBS buffer or pH 3.0 citrate buffer. The yielded solutions were dialyzed for 12 hours in pH 7.4 PBS buffer or pH 3.0 citrate buffer, correspondingly. The dialyzed solution was collected and diluted to 1.0 mL of total volume with addition of pH 7.4 PBS buffer or pH 3.0 citrate buffer, correspondingly. The final **CC/SS-IG** nanoparticle solutions with siRNA (2.31 µM, 0.030 mg/mL) were used for the Quant-iT RiboGreen RNA assay following the manufacturer's protocol.

The loading stability of siRNA inside the **CC/SS-IG** nanoparticles was further evaluated as described in the following. Each **CC/SS-IG** was dissolved to make its final concentration 1.52 mM in DMSO solution containing PEG-lipid at the concentration of 0.616 mM. Under sonication, 150 µL of the above DMSO solution was added dropwise into 450 uL siRNA solution (5.13 µM, 0.067 mg/mL) in 10 mM pH 3.0 citrate buffer. The yielded solution was dialyzed in pH 3.0 citrate buffer for 12 hours and then in pH 7.4 PBS buffer for another 12 hours. The dialyzed solution was collected and diluted to 1.0 mL of total volume with addition of pH 7.4 PBS buffer, yielding the final **CC/SS-IG** nanoparticle solution with siRNA (2.31 µM, 0.030 mg/mL) for the study of the loading stability of siRNA inside the CC/SS-IG nanoparticles with the Quant-iT RiboGreen RNA assay.

The influence of glutathione (GSH) on the siRNA release of IMNA nanoparticles was also evaluated as described in the following manner. GSH was added into IMNA nanoparticle solution in PBS buffer with final 10 mM GSH concentration. The IMNA nanoparticle solution without GSH addition was used a control. After 12 hours, free siRNA in solution was determined with the Quant-iT RiboGreen RNA assay.

The influence of glutathione (GSH) on degradation of SS-DA IMNA nanoparticles was also evaluated as described in the following manner. GSH was added into IMNA nanoparticle solution in DMEM with a final 10 mM GSH concentration. A IMNA nanoparticle dispersion in DMEM without GSH addition was used a control. The nanoparticle solutions were monitored with DLS at a series of time points.

3.4 Evaluation of molecular interaction of siRNA with CC/SS-IGs inside nanoparticles

The interaction of siRNA with each **CC/SS-IGs** within nanoparticles was investigated using JASCO J-815 Circular Dichroism Spectropolarimeter in pH 3.0 citrate buffer and pH 7.4 PBS buffer. Each **CC/SS-IG** was dissolved to make its final concentration 1.52 mM in DMSO solution containing PEG-lipid at the concentration of 0.616 mM. Under sonication, 150 µL of the above DMSO solution was added dropwise into 450 uL siRNA solution (5.13 µM, 0.067 mg/mL) in pH 3.0 10 mM citrate buffer or pH 7.4 PBS buffer. The yielded solutions were dialyzed for 12 hours in pH 3.0 citrate buffer or pH 7.4 PBS buffer, correspondingly. The dialyzed solution was collected and diluted to 800 µL of total volume with addition of pH 7.4 PBS buffer or pH 3.0 citrate buffer, correspondingly. The CD spectrum of the final solutions with siRNA (2.89 µM, 0.038 mg/mL) was recorded by CD. The solutions of free siRNA in pH 3.0 citrate buffer and pH 7.4 PBS buffer at the same siRNA concentrations were used as control.

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3.5 Cellular uptake of CC/SS-IG NPs using confocal microscopy and flow cytometry

Cellular uptake studies were performed using **CC/SS-IGs** that have strong binding affinity with siRNA within nanoparticles in pH 7.4 PBS. HeLa-Luc cells were seeded at a density of 30,000 cells per well in 4-chambered cover glass slides and allowed to attach for 24 hours. NP formulations were prepared by using a similar protocol above with Cy5.5-labeled siRNA. The nanoparticles were added to the cells at a final siRNA dose of 100 nM. After 24 h incubation, the medium was aspirated, washed with PBS, and cell membrane staining was performed (Cell Mask Orange, Molecular Probes) using the manufacturer's protocol. Confocal microscopy imaging was performed using a Zeiss LSM-710 confocal microscopy.

The time-dependent uptake of **CC/SS-IG** nanoparticles was evaluated by flow cytometry after incubation of NPs for different time periods. HeLa cells were seeded in 24-well plates at a density of 150,000 cells/well. After 24 h, culture medium was replaced with fresh medium and NP formulations containing Cy5.5-labeled siRNA were added as described above. After 4, 12 and 24 h of NPs incubation, cells were collected and assayed using BD LSR Fortessa cell analyzer. Data obtained were then analyzed using FlowJo 7.6.5 flow cytometric analysis software.

3.6 Evaluation of *in vitro* gene silencing and cytotoxicity for CC/SS-IG NPs

HeLa cells stably expressing luciferase (HeLa-Luc) were derived from HeLa cells (ATCC) by stable transfection of the firefly luciferase gene using Lentiviral infection followed by clonal selection. HeLa-Luc cells were seeded (10,000 cells/well) into each well of an opaque white 96-well plate (Corning) and allowed to attach overnight in phenol red-free DMEM supplemented with 5% FBS.

The nanoparticles of each **CC/SS-IG** were formulated as described above using firefly luciferase-specific siLuc. The HeLa-Luc cells were transfected with the nanoparticle solution at the dosage of 50, 100, 200, and 400 nM siLuc. Cells were incubated for 36 h at 37 °C, 5% CO_2 and then firefly luciferase activity and viability was analyzed using One Glo + Tox assay kits (Promega). Results were normalized to untreated cells (n=4).

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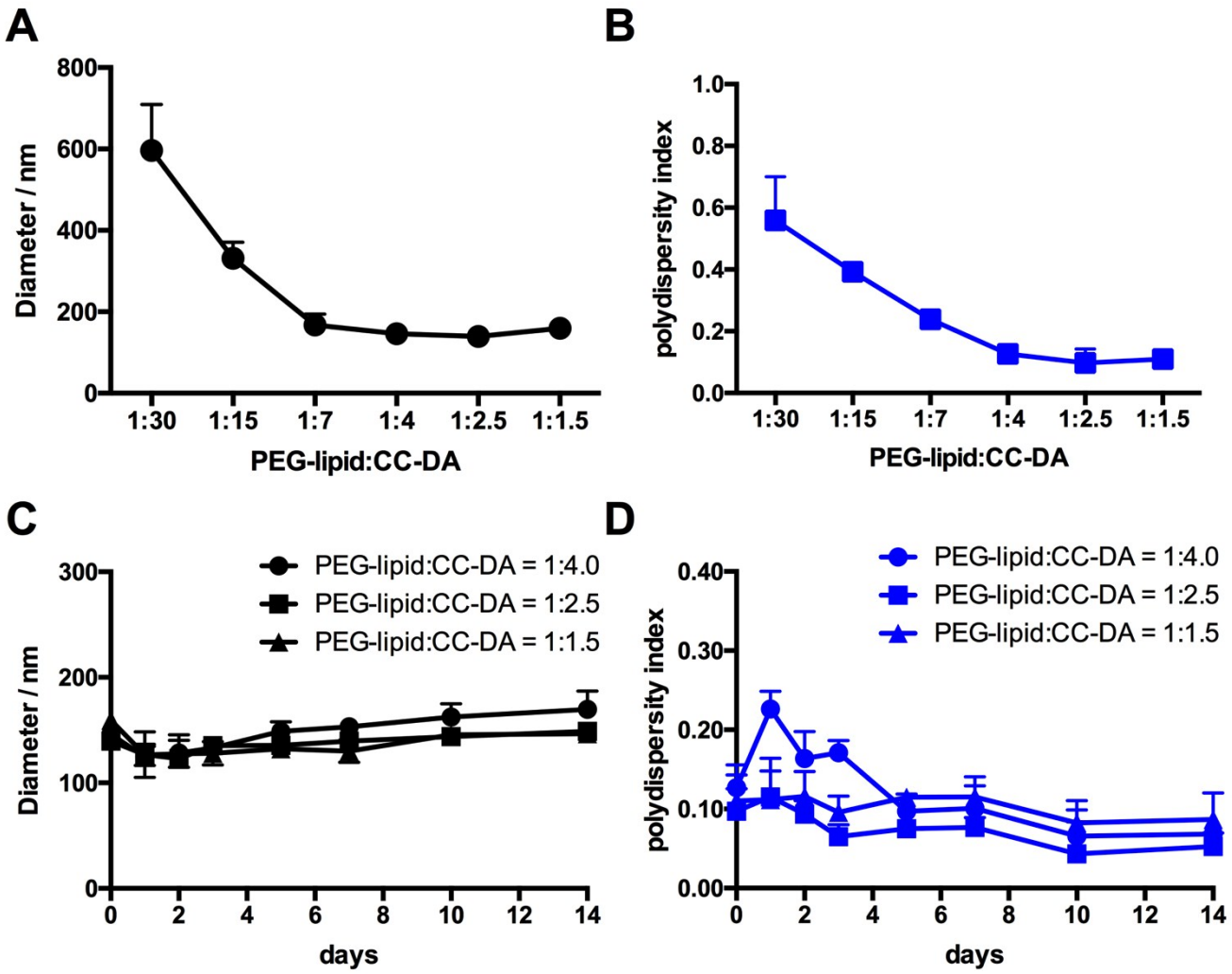

Figure S13. The **CC-DA**/PEG-lipid molar ratio influences the size (**A**) and polydispersity (**B**) of the formulated nanoparticles. The size (**C**) and polydispersity (**D**) of the formulated nanoparticles stays nearly the same for at least two weeks, indicating that stable nanoparticles can be prepared by adjusting the molar ratio of **CC/SS-IG** to PEG-lipid. (N=5 for each data point)

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Table S1. Characterization of formulated CC/SS-IG nanoparticles (NPs)

CC/SS-IGs	Good solvent[a]	Formulated NPs of CC/SS-IGs			
		Apparent pKa[b]	Zeta Potential[c]	Diameter (nm)[d]	PDI[d]
CC-NA	DMSO	4.5	-1.91±0.33[c]	98.06±16.01	0.248±0.009
CC-PY	DMSO	4.2	-0.88±0.13	75.63±1.92	0.085±0.011
CC-DA	DMSO	4.8	-1.20±0.23	124.43±10.11	0.226±0.022
CC-CH	THF	4.0	-1.01±0.36	106.98±10.16	0.187±0.018
CC-DD	DMSO/THF (1/1)	4.1	-1.09±0.17	186.12±13.44	0.200±0.060
SS-NA	DMSO	4.4	-1.56±0.24	101.02±14.32	0.256±0.017
SS-PY	DMSO	4.2	-0.98±0.16	72.57±4.16	0.106±0.029
SS-DA	DMSO	4.9	-1.07±0.33	132.89±12.35	0.196±0.081
SS-CH	THF	3.9	-0.96±0.19	112.05±9.78	0.176±0.052
SS-DD	DMSO/THF (1/1)	4.1	-1.14±0.28	173.77±11.65	0.213±0.043

[a] Solvents were used to dissolve CC-IGs/SS-IGs for nanoparticle formulation. [b]Apparent pKa of each CC/SS-IG in formulated nanoparticles comprised of PEG-lipid/CC-IG or PEG-lipid/SS-IG (1/2.5 mol ratio) was measured by the TNS assay.[c] (mean ± SD, N = 3. [d] mean ± SD, N = 5).

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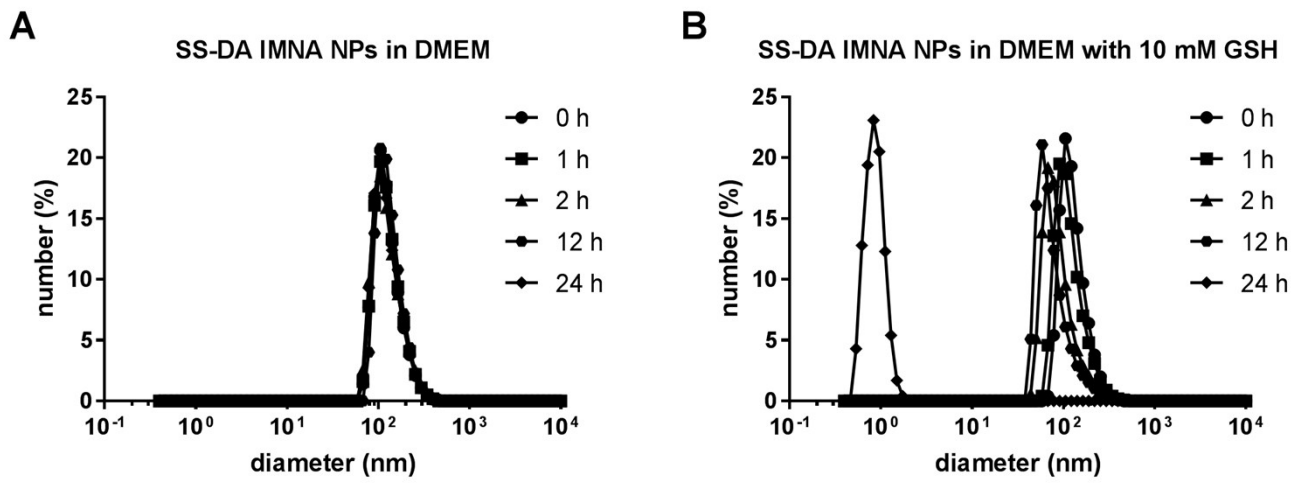

Figure S14. The time dependence of SS-DA IMNA NP diameter in DMEM without (A) and with (B) 10 mM glutathione (GSH).

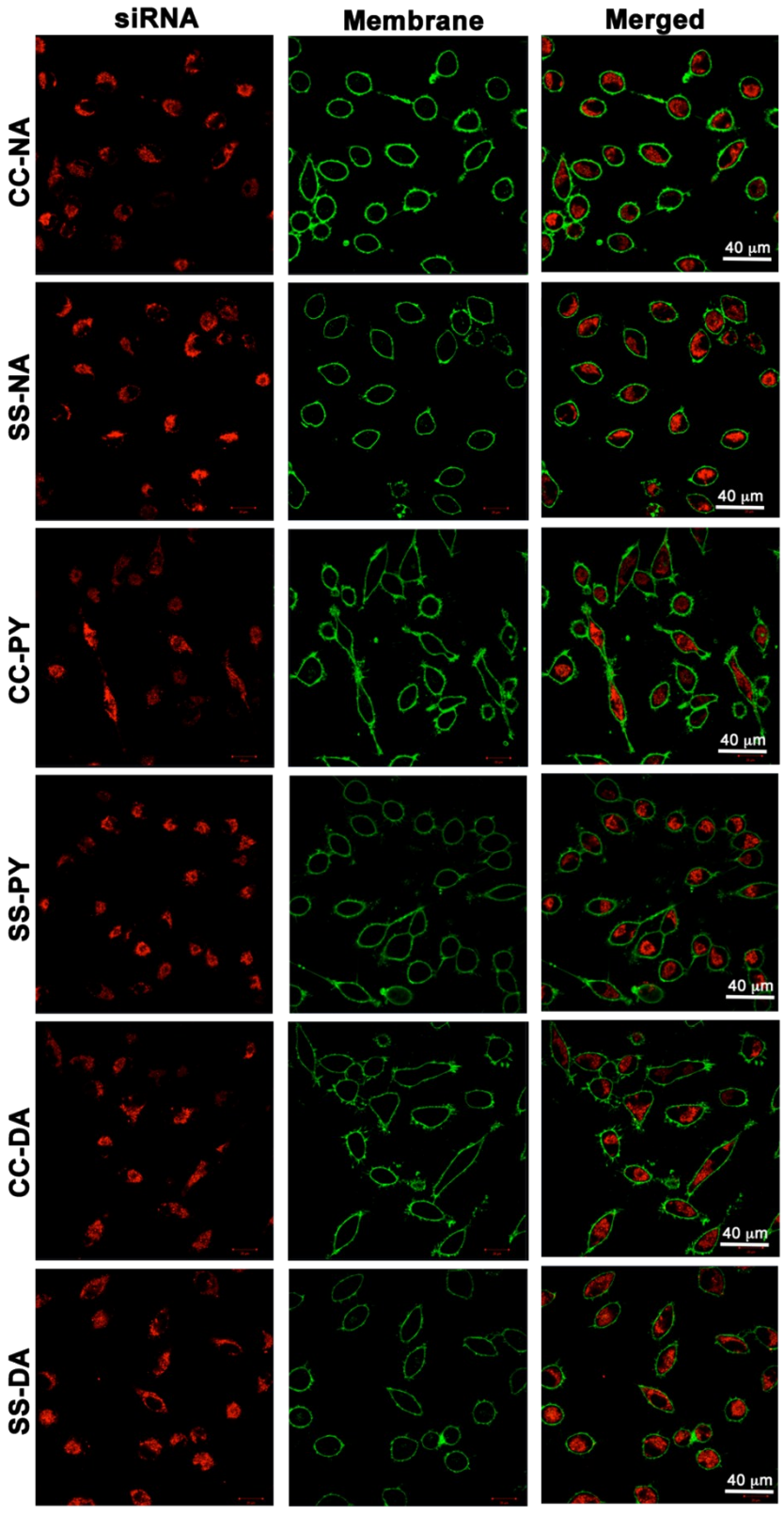

Figure S15. Cellular uptake of formulated Cy5.5-siLuc containing nanoparticles after 24 hour incubation. The nanoparticles are red, and the cell membrane is green (Cell Mask Orange).

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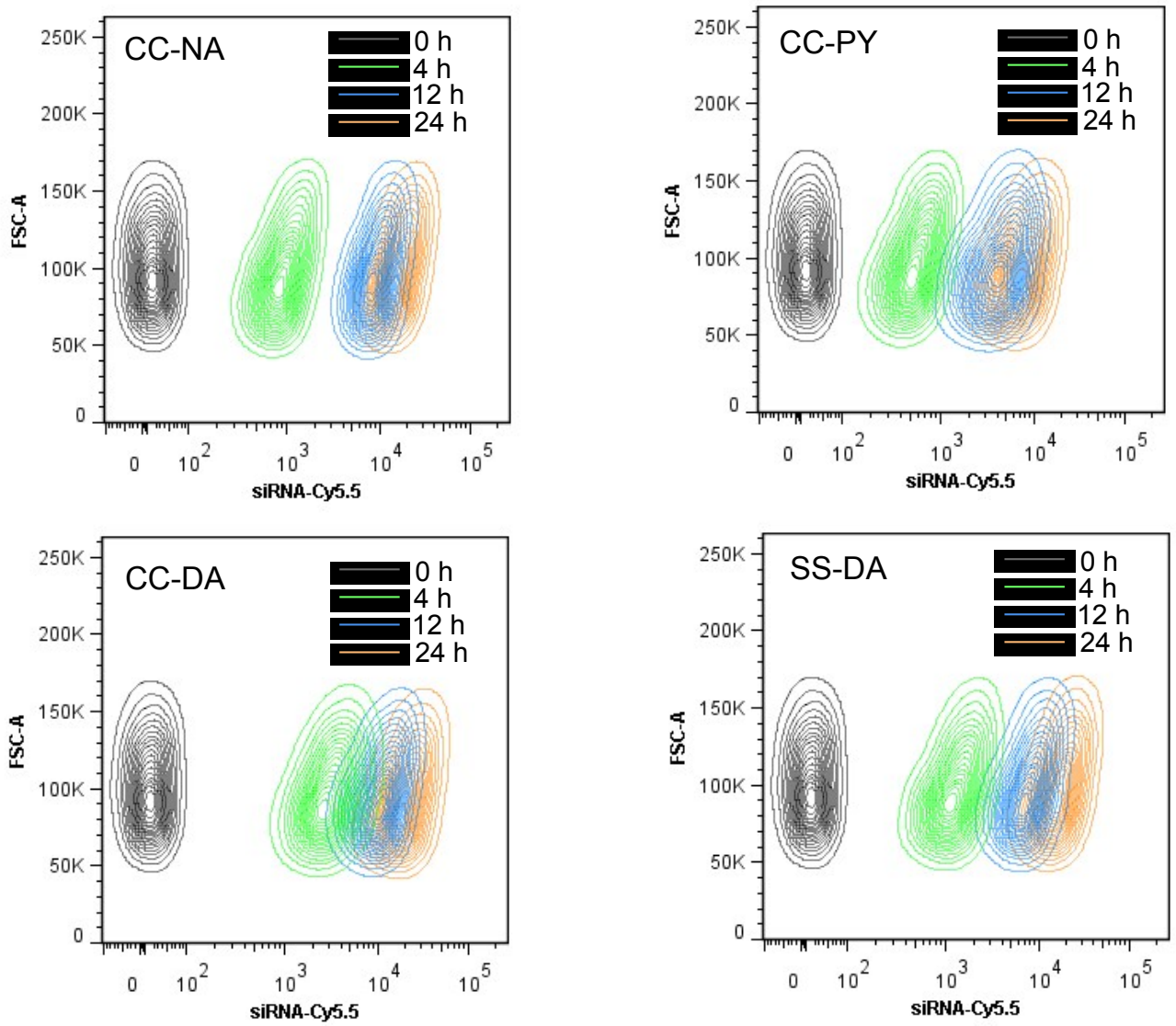

Figure S16. Cellular uptake of formulated Cy5.5-siLuc containing NPs at 0 h (control cells), 4h, 12 h and 24 h.

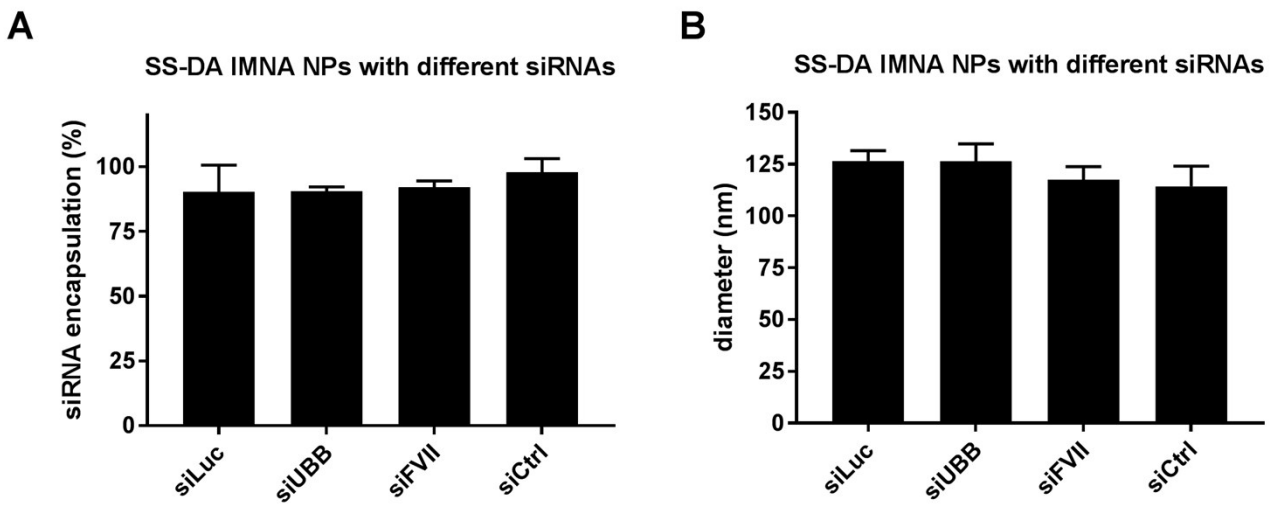

Figure S17. A) Different siRNA sequences (siLuc, siUBB, siFVII and siCtrl) were encapsulated with similar and high efficiency inside SS-DA IMNA NPs in PBS buffer (SS-DA:PEG-lipid:siRNA = 100:40:1 (mol)). B) These SS-DA IMNA NPs have similar size.

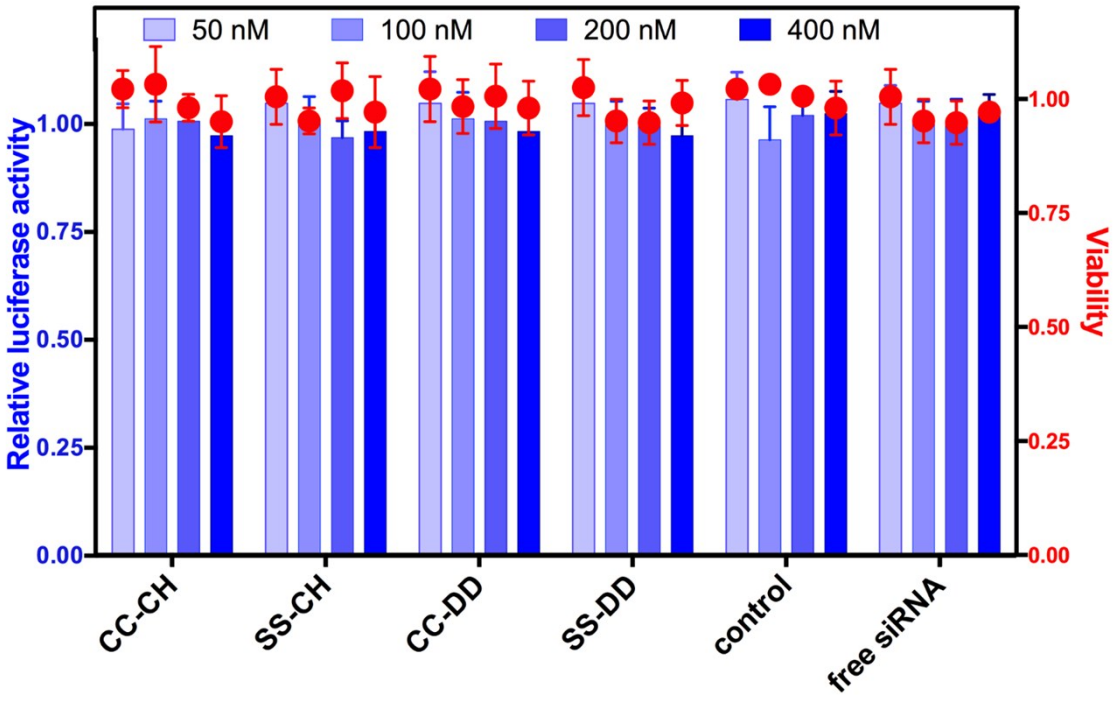

Figure S18. Luciferase knockdown in Hela-Luc cells with the IMNA NPs, PEG-lipid plus siRNA (as control), and free siRNA. Luciferase expression (bars) and viability (dots) were measured after addition of IMNA NPs (50, 100, 200 or 400 nM siRNA, 36 hours incubation) compared to untreated cells. Bars and dots represent mean ± SD (n = 4).

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4. References

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