

Supporting Information

Modular and Hierarchical Self-assembly of siRNAs into Supra-molecular Nanomaterials for Soft and Homogenous siRNA Loading and Precise and Visualized Intracellularly Delivery

Xiaowen Guan, Fanqi Meng, Hongwei Tan, Xiaoni Wang, Jingjing Li,
Juanjuan Wei, Jin Ouyang and Na Na*

Key Laboratory of Radiopharmaceuticals, Ministry of Education, College of
Chemistry, Beijing Normal University, Beijing 100875, China

*Corresponding Authors

Email: nana@bnu.edu.cn

Contents

1. Materials and methods.....	S3
Materials and reagents.....	S3
Apparatus.....	S3
Preparation of WP5⊃G nanoparticles.....	S3
Prepared siRNA loaded vesicles.....	S4
Transmission electron microscopy (TEM).....	S4
Cell culture.....	S4
In vitro cell cytotoxicity assay.....	S4
Confocal laser scanning microscopy (CLSM).....	S5
In Vivo Imaging and Antitumor Assessment.....	S5
In vivo Imaging.....	S5
Tumor growth inhibition.....	S5
2. Synthesis of host WP5.....	S6
3. Synthesis of guest G.....	S10
Synthesized of compound 2.....	S10
Synthesized of compound G.....	S12
4. DLS and TEM data.....	S14
5. NMR spectra of WP5 and G.....	S19
6. siRNA loading.....	S23

1. Materials and methods

Materials and reagents

All reagents were purchased from commercial suppliers and used without further purification unless specified. Water used in this work was triple distilled. Fetal bovine serum (FBS), penicillin-streptomycin and PBS were purchased from Invitrogen (Carlsbad, CA, USA). Minimum Eagle's medium (MEM) and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco (Thermo Fisher Scientific). Cell culture was carried out in an incubator with a humidified atmosphere of 5 % CO₂ at 37 °C. MRP1 siRNA, and FAM-MRP1 siRNA were custom-synthesized by Sangon Biological Co. Ltd (Shanghai, China). The respective siRNA sequences are listed below.

siRNA: sense 5'-GGCUACAUCAGAUGACACdTdT-3',

antisense 5'-GUGUCAUCUGAAUGUAGCCdTdT-3',

FAM-siRNA: sense 5'-FAM-GGCUACAUCAGAUGACACdTdT-3',

antisense 5'-GUGUCAUCUGAAUGUAGCCdTdT-3',

Apparatus

¹HNMR and ¹³CNMR spectra were obtained on a JEOL-600 (or JEOL-400) spectrometer (JEOL, Japan). MS data were recorded on an LTQ-XL mass spectrometer (Thermo Scientific, USA) under positive mode, equipped with the ion source of electrospray ionization (ESI) and ambient pressure chemical ionization (APCI). The transmission electron microscopy (TEM) images were obtained with a FEI Talos F200s TEM (Thermo Fisher Scientific, USA). Fluorescence (FL) spectra were collected by FS-5 fluorescence spectrophotometer (Edinburgh instruments, UK). 50 μL quartz fluorescence cuvette was purchased from Yixing Purshee Optical Elements Co., Ltd (Zhejiang, China). UV-visible spectra were collected on a UV2600 spectrophotometer (Shimadzu, Japan). Dynamic light scattering (DLS) was carried out on a Nano-ZS Zetsozer ZEN3600 (Malvern Instruments Ltd., U.K.). Water surface tension was recorded with BZY-3B surface tension measurer (China). Confocal laser scanning microscopy (CLSM, Nikon A1R, Japan) was used for cell imaging.

Preparation of WP5⊃G nanoparticles

WP5 (100 μmol), G (50 μmol) were dissolved in 25 mL deionized water, and after ultrasonic for 30 minutes and standing overnight. Then, the prepared WP5⊃G nanoparticles were purified by dialysis (molecular weight cutoff 8000-10000) in deionized water for several times until the water outside the dialysis tube does not appear red.

Prepared siRNA loaded vesicles

First, G (50 μmol) and FAM-siRNA or siRNA (10 μmol) were dissolved in 2 mL water, and the mixture solution was stirred 15 min. Then, WP5 (100 μmol) were added above mixed solution, and after ultrasonic for 30 minutes and standing overnight. Then, the prepared WP5 \supset G- siRNA nanocapsules were purified by dialysis in deionized water for several times until the water outside the dialysis tube does not appear red. The same procedure was used for loading of siRNA into the vesicles.

Transmission electron microscopy (TEM)

TEM was carried out on a FEI Tecnai G2 Spirit BioTwin TEM with an accelerating voltage of 120 kV and a FEI Talos F200s TEM (Thermo Fisher Scientific, USA). The TEM samples were prepared by depositing one drop of the sample solutions onto carbon-coated copper grids, and the grids were dried in the air at room temperature.

Cell culture

293T and SKOV-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10.0% fetal bovine serum (FBS) and 1.00% penicillin/streptomycin. After the cells grew as a monolayer and were detached upon confluence using trypsin (0.5% w/v in PBS), they were harvested from cell culture medium by incubating in trypsin solution for 5 min. Then, the cells were centrifuged at 3000 rpm for 5 min, and the supernatant was discarded. A 3.00 mL portion of serum-supplemented DMEM was added to neutralize any residual trypsin. Finally, the cells were resuspended in serum-supplemented DMEM at a concentration of 1.00×10^4 cells per mL. Cells were cultured at 37 °C and 5.00% CO₂.

In vitro cell cytotoxicity assay.

The cytotoxicity is evaluated by the cellular viability by CCK assay. All the solutions were sterilized by filtration with a 0.22 μm filter before tests. 293T and SKOV-3 Cells were seeded at a density of 1.00×10^4 cells per well in a 96-well plate, and incubated for 24 h for attachment. Then, cells were incubated with WP5 \supset G at various concentrations for 24 h, respectively. CCK solution (20 μL , 5 mg/mL) is added to each well and incubated at 37 °C for another 4 h. The absorbance of each well at 450 nm (620 nm wavelength as reference) is measured by a multimode microplate reader (Tecan infinite 200). The cytotoxicity assays are shown using mean SD (n = 3).

Confocal laser scanning microscopy (CLSM).

SKOV-3 cells and 293T cells were seeded in 35 mm glass-bottomed Petri dishes for 24 h. The medium was replaced with fully supplemented Dulbecco's Modified Eagle Medium (DMEM). The cells were then incubated with WP5 \supset G and WP5 \supset G-FAM-siRNA complexes for 30 min, respectively. The dishes were then washed with PBS three times and fixed with 4.0% paraformaldehyde at room temperature for 15 min. After washing with PBS, the cells were all stained with Hoechst 33258 for 10 min. Finally, the cells were washed with PBS three times and then observed under a confocal fluorescence microscope (Nikon A1R).

In Vivo Imaging and Antitumor Assessment

All animal experiments were conducted in accordance with the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Ethics Committee of Beijing Normal University. SKOV-3 cells (5×10^6 cells per mouse) were injected subcutaneously into the right front legs of nude mice to establish the SKOV-3 tumor xenograft-bearing models.

In vivo Imaging.

For in vivo imaging, the mice were injected with WP5 \supset G+FAM-siRNA vesicles by the tail vein and fed for approximately another 6 h. Before imaging experiments, the mice were anesthetized and fixed in the imaging system. Next, the mice were examined by the Caliper IVIS Lumina III imaging system, with 480 nm excitation (green imaging) for siRNA detection and 540 nm excitation (red imaging) for WP5 \supset G detection.

Tumor growth inhibition.

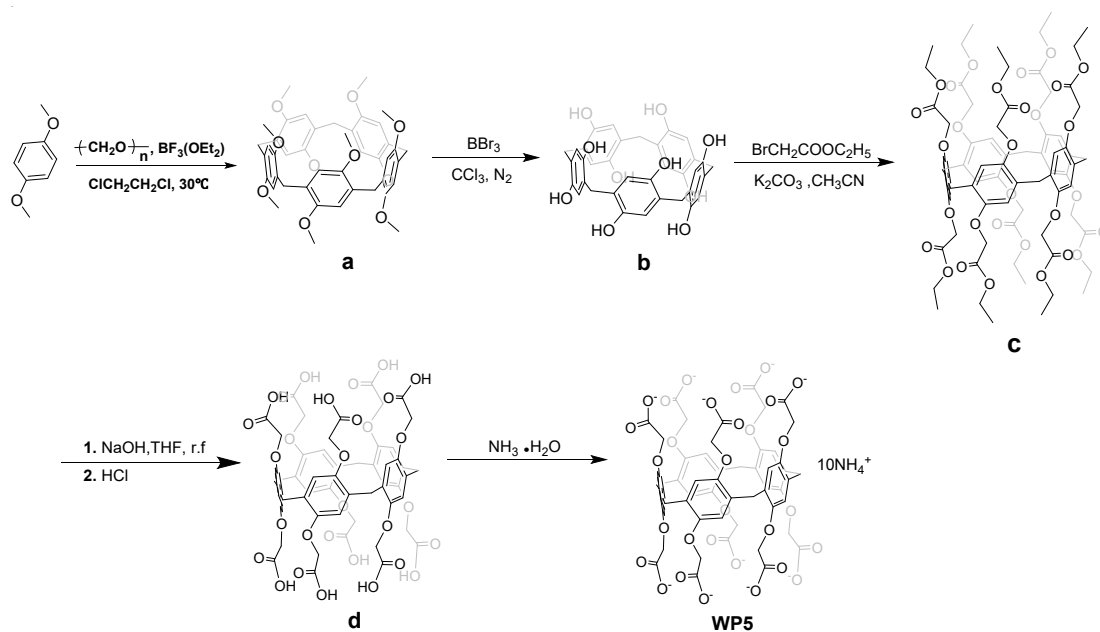
For in vivo antitumor study, the mice were randomly divided into three groups ($n = 5$) for treatment when the tumor volume reached about average size of 100-120 mm³. 200 μ L of therapeutic nanoagents (1 mg kg⁻¹ siRNA) was injected into each nude mouse via tail vein every other day for 14 days and meanwhile tumor weight and size were monitored. Tumor size was measured by a caliper and tumor volume calculated according to the following formula:

$$\text{tumour volume(mm}^3\text{)} = \text{length} \times \text{width}^2 / 2$$

After 14th therapy, tumors and major organs were sectioned for hematoxylin-eosin staining (H&E) analyses.

2. Synthesis of host WP5

The synthetic procedures for host **WP5** was shown in **Scheme S1**.



Scheme S1. Synthetic route for host **WP5**.

Compound **a** was prepared according to previously reported method.^{S1}

^1H NMR (400 MHz, CDCl_3 , 25°C) δ (ppm): 6.79 (s, 10H), 3.77 (s, 10H), 3.67 (s, 30H).

^{13}C NMR (150 MHz, CDCl_3 , 25°C): δ 150.60, 128.18, 113.80, 55.64, 29.43.

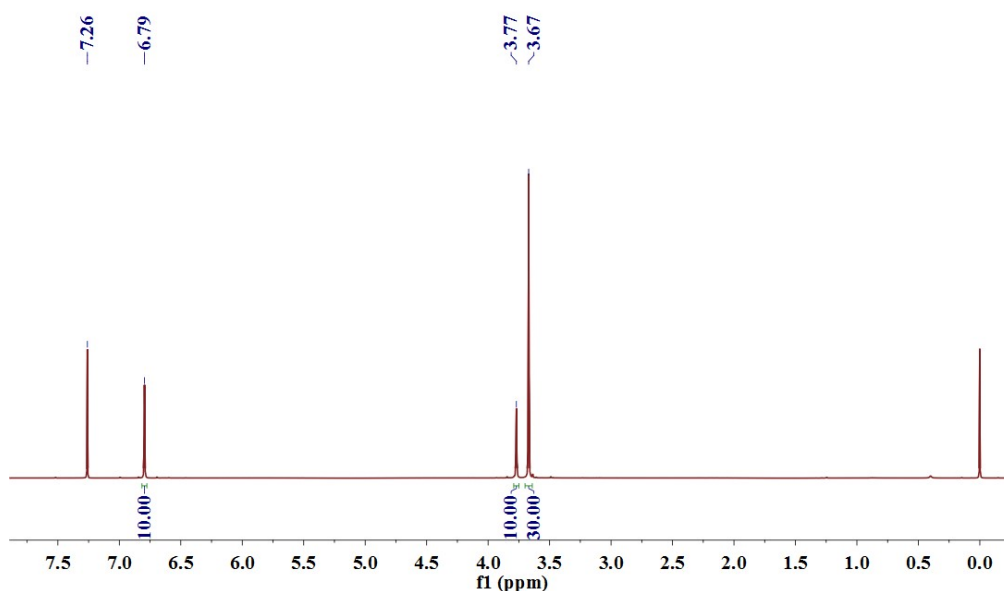


Figure S1. ^1H NMR spectrum of **a** in CDCl_3 .

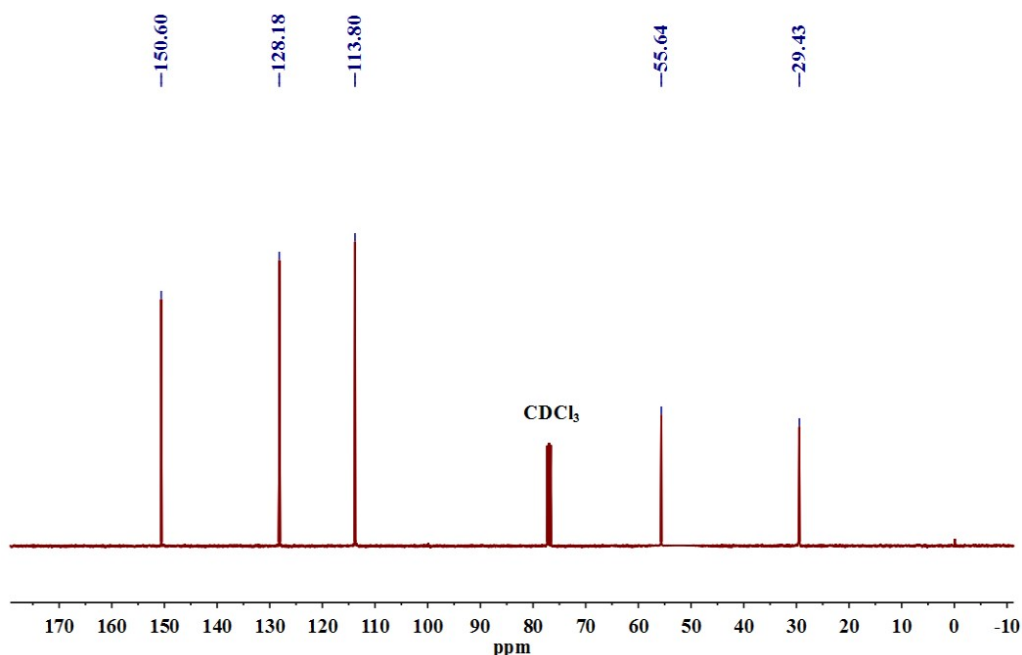


Figure S2. ¹³C NMR spectrum of **a** in CDCl₃.

Compound b and c were prepared according to previously reported method. ^{S1,S2}

To a solution of **a** (1.5 g, 2 mmol) in dry CHCl₃ (60 mL), boron tribromide (24.3 g, 97.3 mmol) was added in the condition of ice bath, and the mixture was stirred at room temperature for 72 h. Then, 60 mL water was added into the mixture, which was stirred for 3 h. The precipitate formed was filtered and washed with water to result in white solid **b** (1.2 g, 96% yield).

Compound **b** (1.22 g, 2.0 mmol) was dispersed in MeCN (50 mL) and K₂CO₃ (3.5 g) was added. The mixture was stirred for 30 min at room temperature, then a small amount of KI and excess of ethyl bromoacetate (5.0 mL, 45 mmol) were added. The mixture was heated under reflux under a nitrogen atmosphere for 24 h, and then filtered and washed with CH₂Cl₂ after cooling down. The filtrate was concentrated, and the residue was subjected to column chromatography on silica gel to give the **c** as white solid (2.35 g, 80% yield). ¹H NMR (600 MHz, CDCl₃, 298 K) δ (ppm): 7.03 (s, 1H), 4.58-4.48 (m, 20H), 4.10-4.03 (m, 20H), 3.84 (s, 10H), 0.97-0.93 (t, *J* = 6 Hz, 30H). ¹³C NMR (150 MHz, CDCl₃, 25 °C): δ 169.40, 149.04, 128.76, 114.52, 65.78, 60.94, 29.29, 13.90.

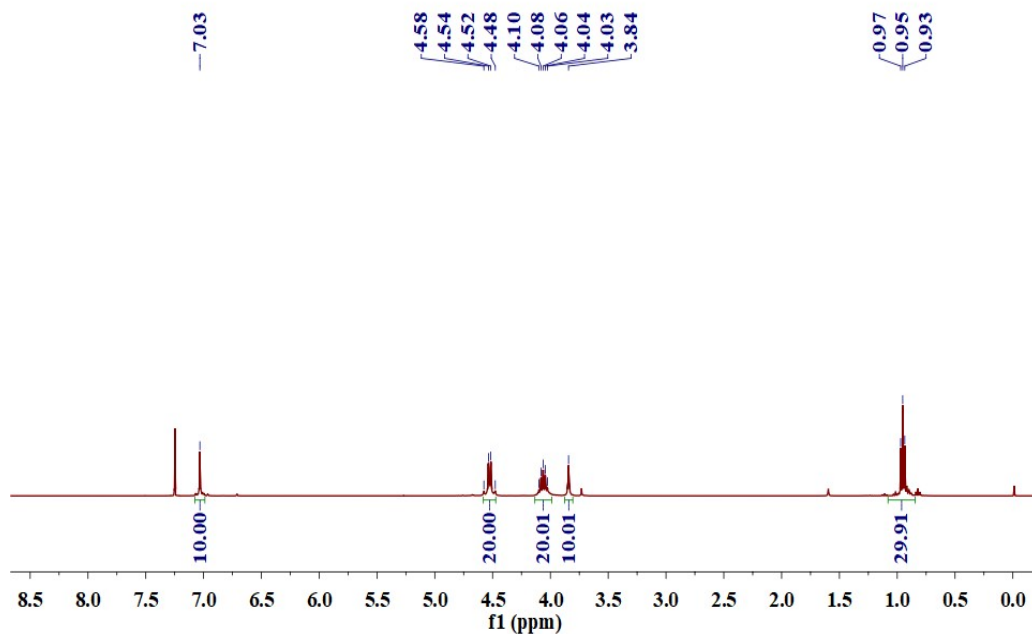


Figure S3. ^1H NMR spectrum of **c** in CDCl_3

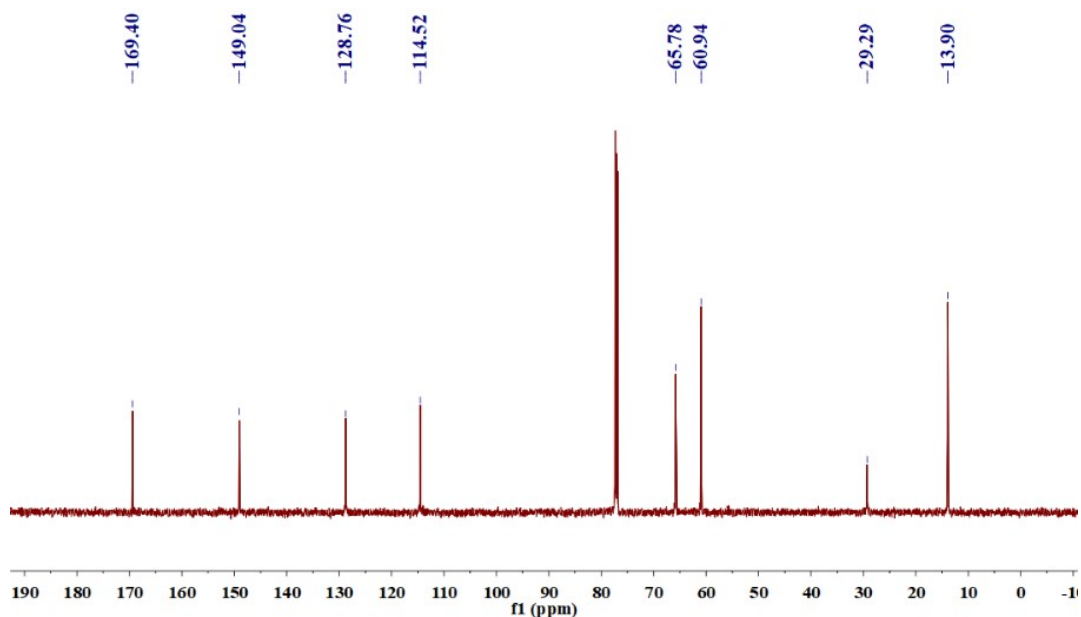


Figure S4. ^{13}C NMR spectrum of **c** in CDCl_3 .

Compound **d** was synthesized and purified according to previously reported procedures.^{S1-S3}

NaOH solution (30%, 45 mL) was added to a solution of **c** (1.47 g, 1 mmol) in THF (70 mL). The mixture was heated under reflux for 15 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. The residue was diluted into 100 mL of H_2O , and then acidified with HCl. The resulting precipitate was collected by filtration, and dried under vacuum to give white solid **d**. Yield: 1.1 g, 92.4%. ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 298 K) δ (ppm): 12.91 (s, 10H), 7.05 (s, 10H), 4.69 (d, $J=4.65$ Hz, 10H),

4.41 (d, $J=4.37$ Hz, 10H), 3.69 (s, 10H).

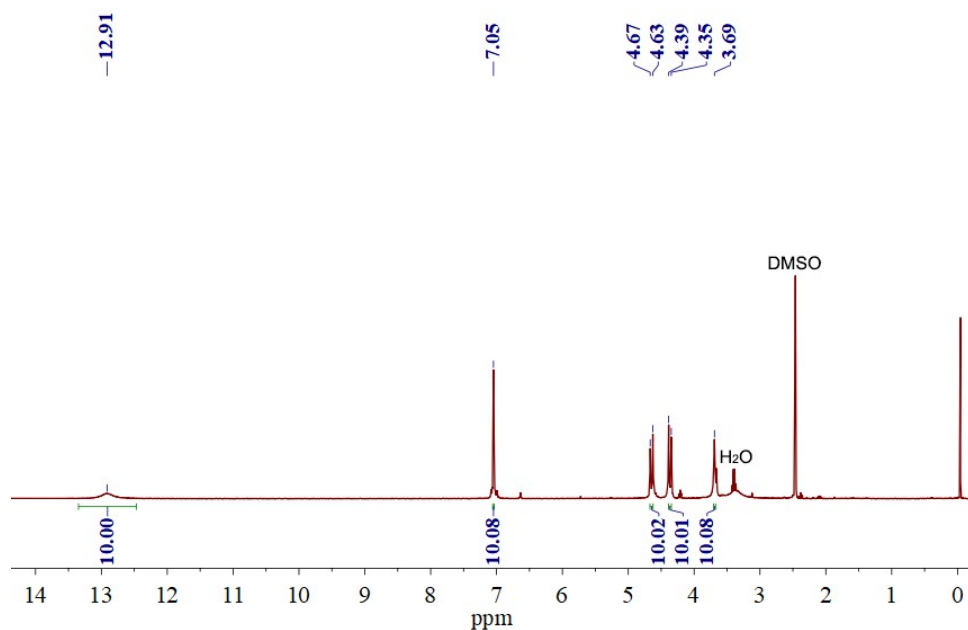


Figure S5. ^1H NMR spectrum (400 MHz) of **d** in $\text{DMSO-}d_6$.

Compound **WP5** was synthesized and purified according to previously reported procedures.^{S3, S4}

^1H NMR (600 MHz, D_2O , 298 K) δ (ppm): 6.61 (s, 10H), 4.11 (s, 10H), 3.74 (s, 10H).

^{13}C NMR (150 MHz, D_2O , 298 K): δ 177.42, 149.53, 128.89, 114.93, 68.15, 29.11.

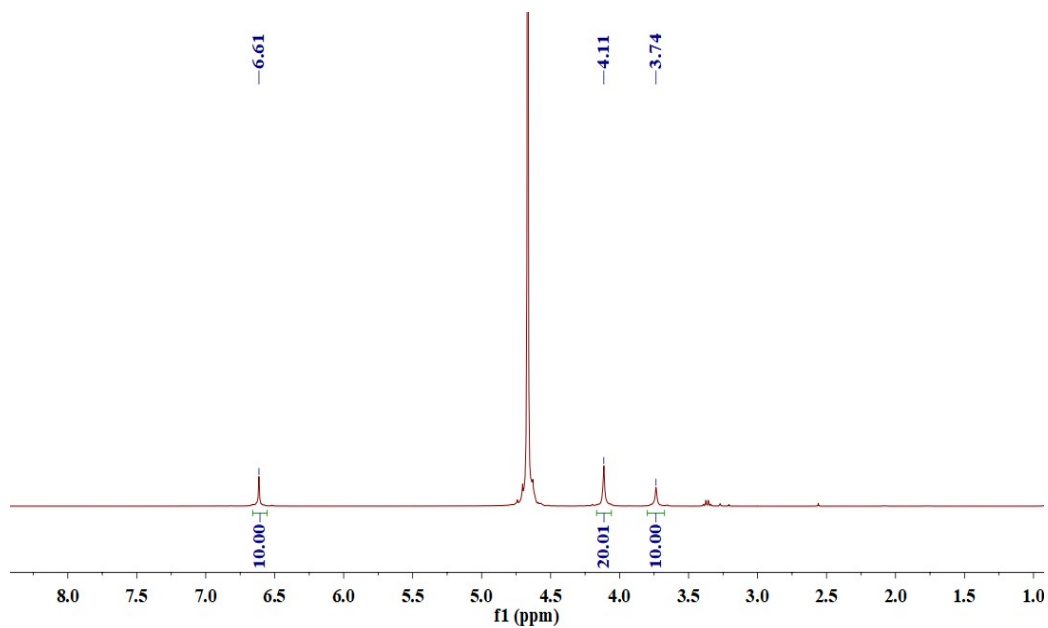


Figure S6. ^1H NMR spectrum (400 MHz) of **WP5** in D_2O .

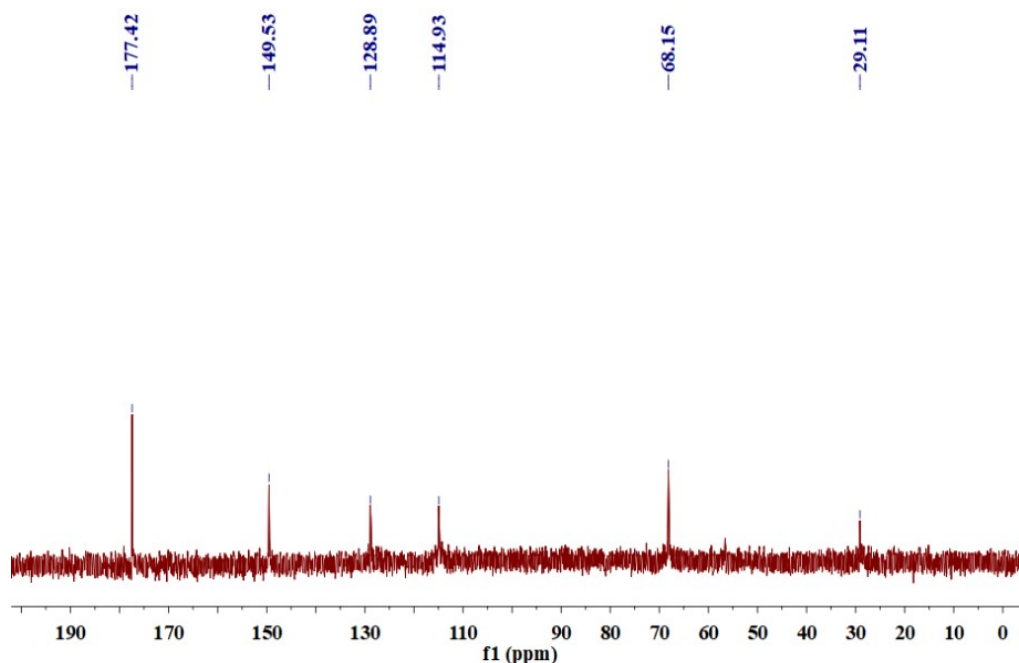
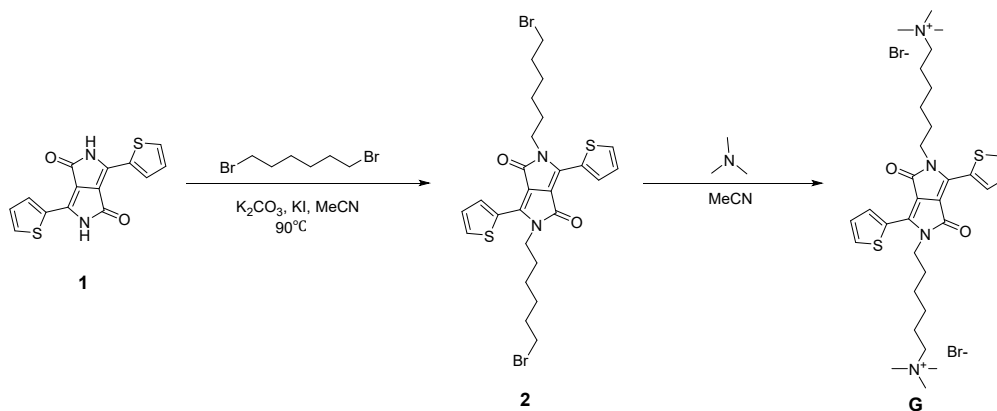


Figure S7. ^{13}C NMR spectrum (400 MHz) of **WP5** in D_2O .

3. Synthesis of guest **G**

The synthetic procedures for guest **G** were shown in **Scheme S2**.



Scheme S2. Synthetic route for guest **G**.

Synthesized of compound **2**.

Compound **1** (0.60 g, 2.0 mmol), 1,6-dibromohexan (1.46 g, 6.0 mmol) and Cs_2CO_3 (3.4 g, 10.0 mmol) is dissolved into MeCN (50 mL) and stirred at 85 °C overnight. After cooling, the mixture was partitioned between water (100 mL) and dichloromethane (100 mL). The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. Chromatographic purification (petroleum ether/dichloromethane, 2:1, v/v) and drying under vacuum afforded a purple solid **2** (0.81 g, 65%). ^1H NMR (600MHz, CDCl_3 , 298 K): δ (ppm) 8.91 (dd, $J = 8.9$ Hz, 2H), 7.64-7.63 (dd, $J = 7.6$ Hz,

2H), 7.29-7.27 (m, 2H), 4.09-4.06 (m, 4H), 3.40-3.37 (t, $J = 3.38$ Hz, 4H), 1.89-1.82 (m, 4H), 1.79-1.72 (m, 4H), 1.51-1.43 (m, 8H). ^{13}C NMR (150 MHz, CDCl_3 , 298 K): δ 161.43, 140.06, 135.44, 130.85, 129.73, 128.78, 107.75, 42.06, 33.81, 32.67, 29.84, 27.86, 26.11.

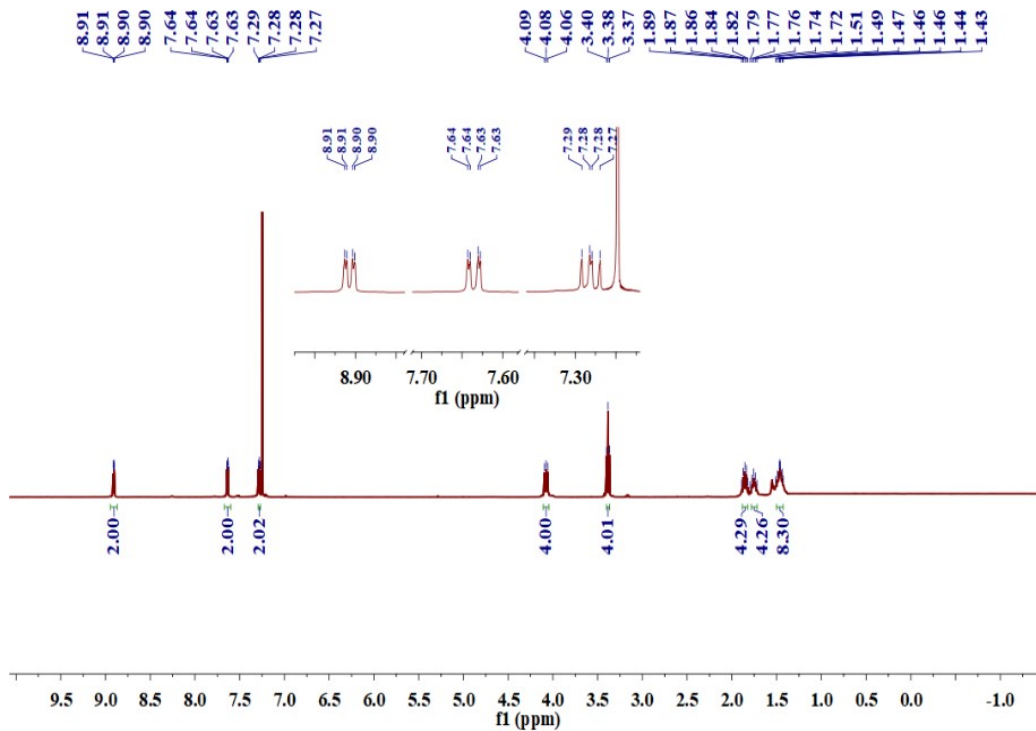


Figure S8. ^1H NMR spectrum (400 MHz) of **1** in CDCl_3 .

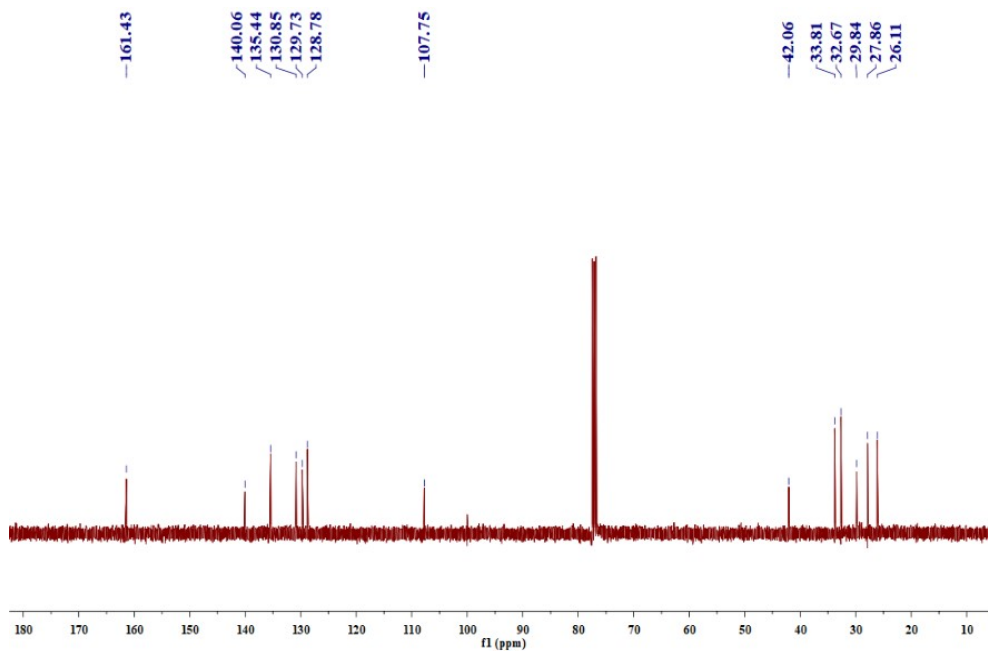


Figure S9. ^{13}C NMR spectrum of **2** in CDCl_3 .

Synthesized of compound G.

To a stirred solution of compound **2** (0.63 g, 1.0 mmol) in MeCN (45 mL) at room temperature was added trimethylamine (33.0% in ethyl alcohol, 8.00 mL). The resulting solution was refluxed for 24 h in a sealed flask. After removal of the solvent, anhydrous diethylether was added to give precipitate **G** as a fuchsia solid (0.71 g, 96.0%). ^1H NMR (600 MHz, D_2O): δ 8.27-8.26 (d, $J = 8.3$ Hz, 2H), 7.70-7.69 (d, $J = 7.7$ Hz, 2H), 7.12-7.10 (t, $J = 7.1$ Hz, 2H), 3.54-3.50 (m, 4H), 3.14-3.10 (t, $J = 7.2$ Hz, 4H), 3.01-2.96 (m, 18H), 1.59-1.56 (m, 4H), 1.38-1.35 (m, 4H), 1.17 (s, 4H), 1.08-1.04 (m, 4H). ESI-MS m/z : $[\text{M} - 2\text{Br}]^{2+}$ calcd for $\text{C}_{32}\text{H}_{48}\text{N}_4\text{O}_2\text{S}_2$ 292.1604; found, 292.1667.

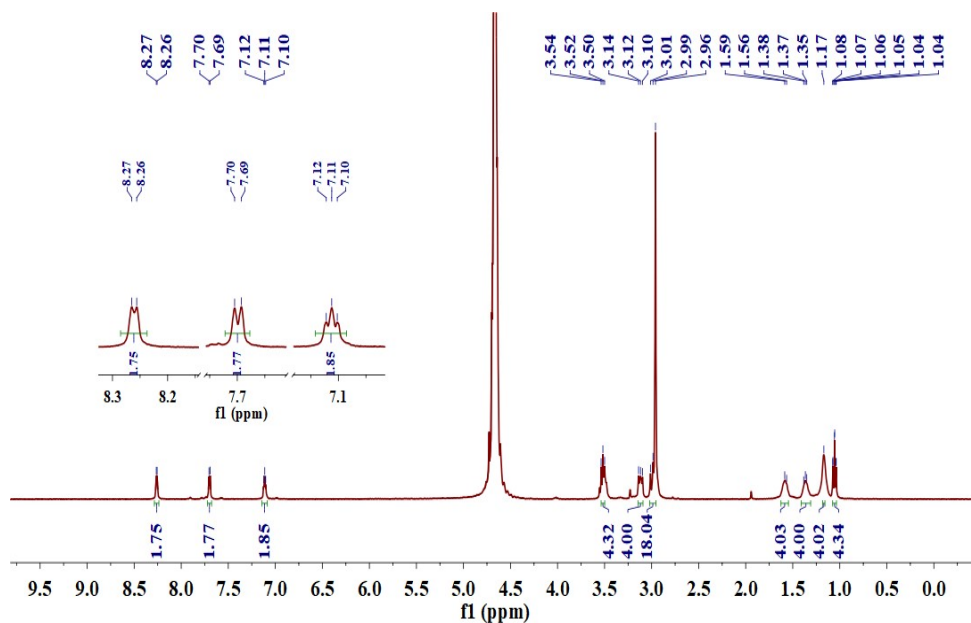


Figure S10. ^1H NMR spectrum (400 MHz) of **G** in D_2O .

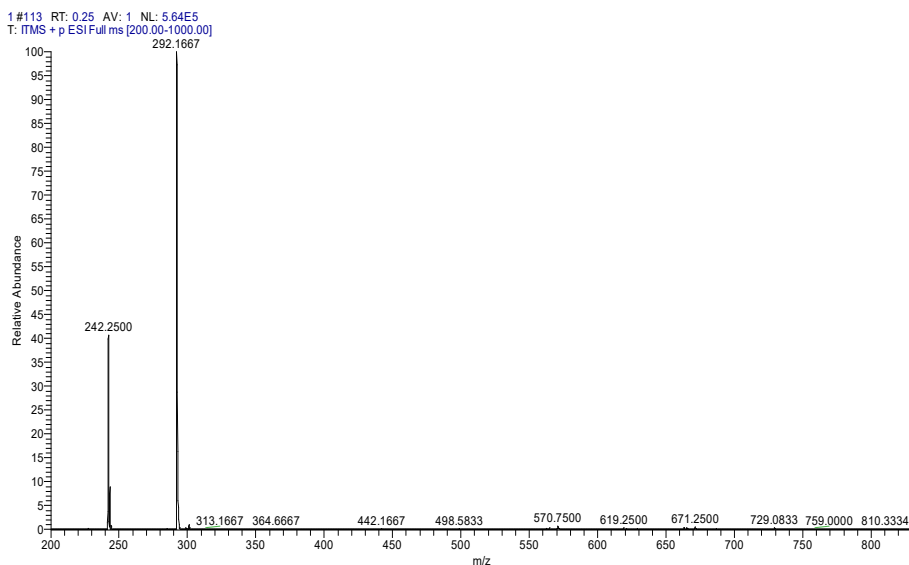


Figure S11. MS spectrum of **G**.

Reference

- S1.** H. Li, D.-X. Chen, Y.-L. Sun, Y. B. Zheng, L.-L. Tan, P. S. Weiss, Y.-W. Yang, *J. Am. Chem. Soc.* 2013, **135**, 1570–1576.
- S2.** Y. Chang, K. Yang, P. Wei, S. Huang, Y. Pei, W. Zhao, Z. Pei, *Angew. Chem. Int. Ed.* 2014, **53**, 13126–13130.
- S3.** B. Li, Z. Meng, Q. Li, X. Huang, Z. Kang, H. Dong, J. Chen, J. Sun, Y. Dong, J. Li, X. Jia, J. L. Sessler, Q. Meng, C. Li, *Chem. Sci.*, 2017, **8**, 4458-4464.
- S4.** G. Sun, Z. He, M. Hao, M. Zuo, Z. Xu, X.-Y. Hu, J.-J. Zhu, L. Wang, *J. Mater. Chem. B*, 2019, **7**, 3944–3949.

4. DLS and TEM data WP5 \supset G-siRNA.

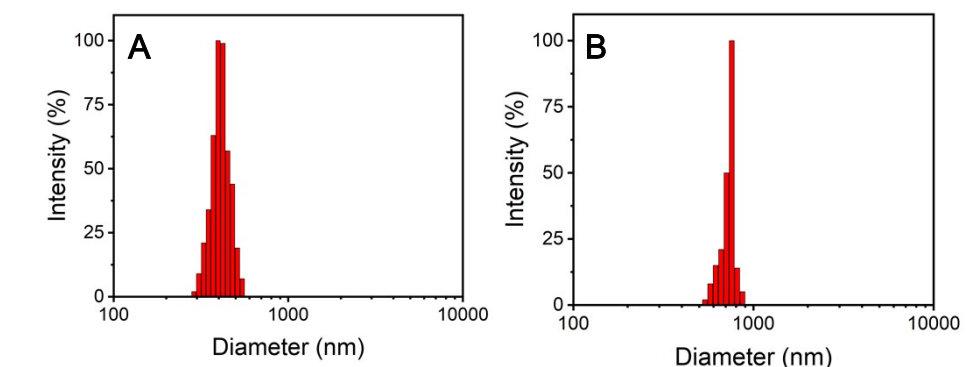


Figure S12. The size of WP5 \supset G-siRNA with the core of G-siRNA assembled in PBS (PH=7.0) for different times. (A) WP5 was added after 12 hours of assembly between G and siRNA. (B) WP5 was added after 24 hours of assembly between G and siRNA.

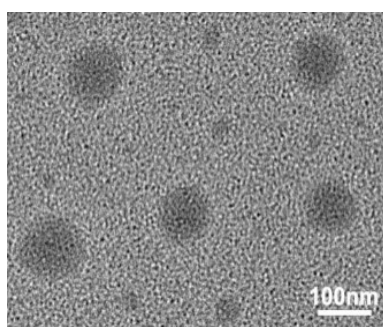


Figure S13. TEM image of G-siRNA.

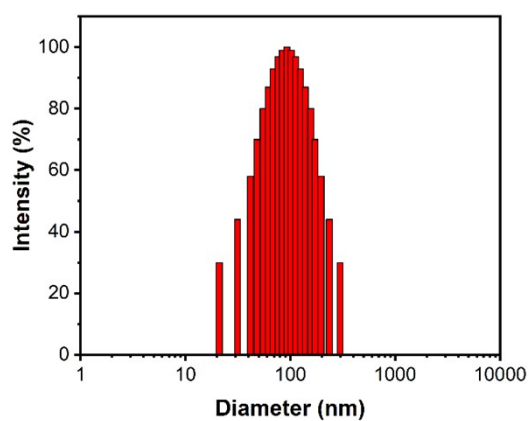


Figure S14. DLS data of G-siRNA.

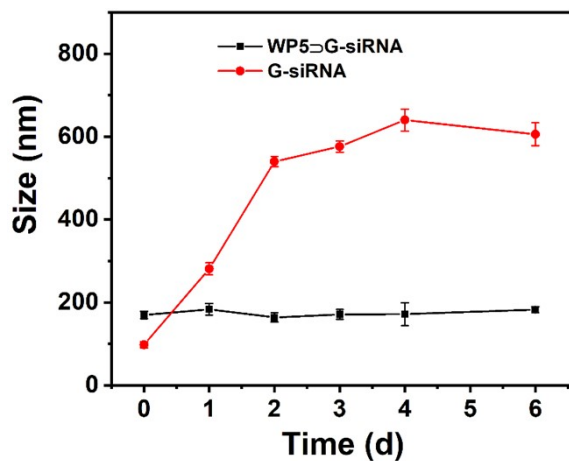


Figure S15. Time-dependent DLS variations of **G-siRNA** with and without **WP5** over time recorded on two samples in PBS (PH=7.0) for 6 days as determined.

The increase of G-siRNA size during the first 2-4 days could be generated from the multiple interactions not only include the assembly between G and siRNA, but also involved self-assembly of G through π - π stacking and cation- π interactions. Therefore, the size of G-siRNA increased with increasing interaction time during the first 4 days. While no obvious size increase was observed after 4th day, because the relative weak interactions would be hard to maintain the larger structures.

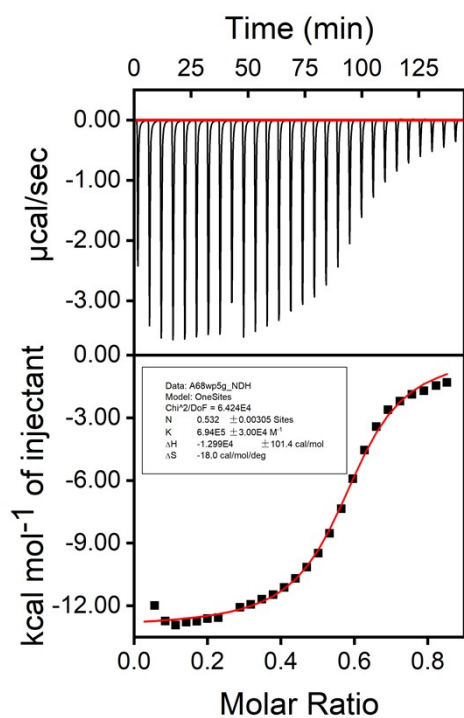


Figure S16. ITC measurement on binding of WP5 with G at 298 K, pH = 7.0. Raw ITC data were obtained by 28 sequential injections of a WP5 solution (1.00 mM) into a G solution (0.2 mM).

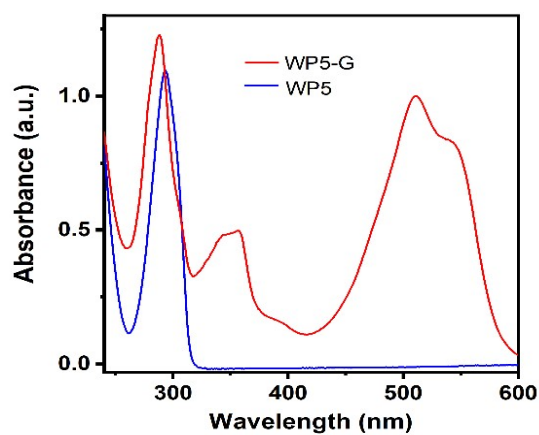


Figure S17. Absorbance spectra of G (1.00 × 10⁻⁴ M) in aqueous without and in the presence of WP5 (2.00 × 10⁻⁴ M) at 298 K. Wavelength range: 220 nm-650 nm.

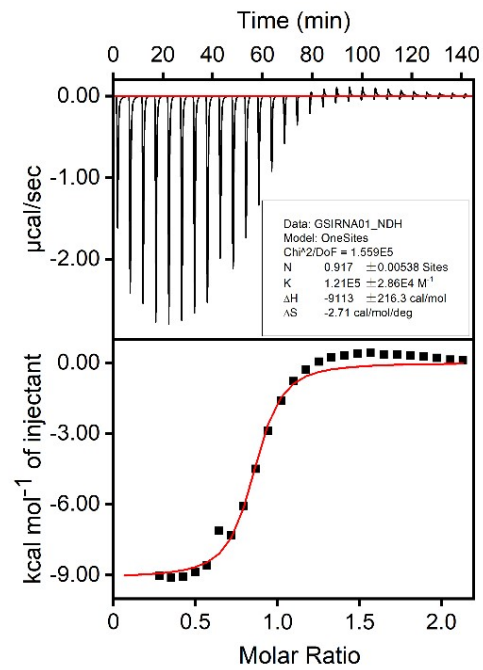


Figure S18. ITC experiments of WP5 with G-siRNA in aqueous solution at 298 K. Raw ITC data for sequential injections of a WP5 solution (1.00 mM) into a G-siRNA solution (0.1 mM).

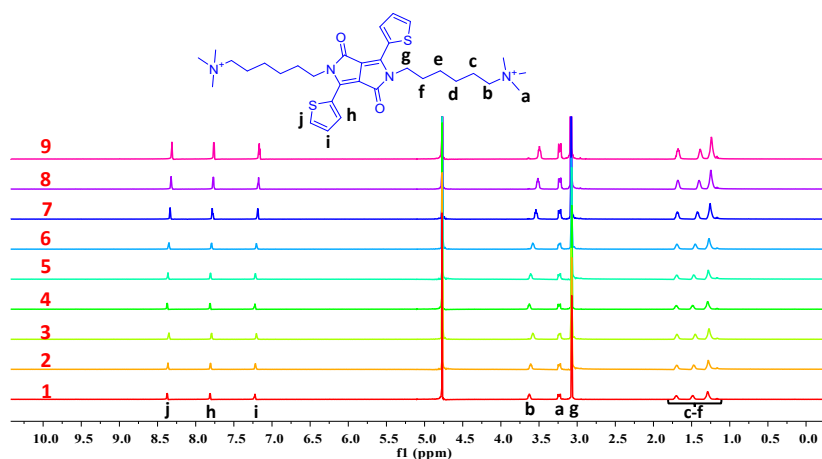


Figure S19. ^1H NMR spectra of G at different concentrations (mM): (1) 8.0, (2) 10.0, (3) 12.0, (4) 16.0, (5) 20.0, (6) 28.0, (7) 36.0, (8) 44.0, (9) 52.0 (600 MHz, D_2O , 298 K).

With the concentrations increase, the signals of protons Hb-f show obvious upfield shifts, revealing the hydrophobic interactions among the long alkyl chains of adjacent G molecules. Meanwhile, the signals of Hh-j also upfield shifts, which indicates that π - π stacking interactions between diketopyrrolo-pyrrole-thiophene appear during the self-assembly of G.

5. NMR spectra of WP5 and G

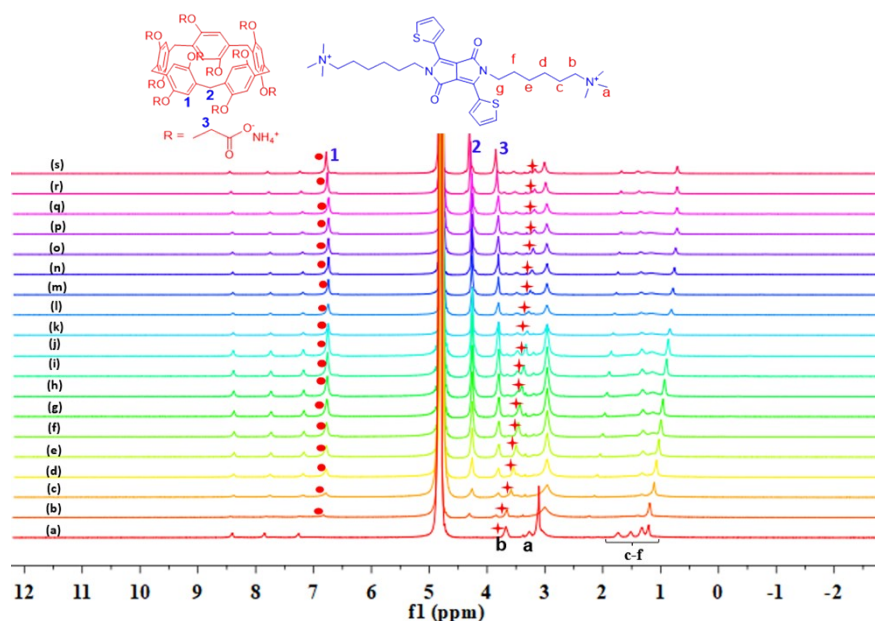


Figure S20. ^1H NMR spectra of G at a constant concentration of 8.0 mM with different concentrations of WP5 (mM): (a) 0.0, (b) 1.0, (c) 2.0, (d) 3.0, (e) 4.0, (f) 5.0, (g) 6.0, (h) 7.0, (i) 8.0, (j) 9.0, (k) 10.0, (l) 11.0, (m) 12.0, (n) 13.0, (o) 14.0, (p) 15, (q) 16.0, (r) 17.0, (s) 18.0 (600 MHz, D_2O , 298 K).

Table 1. Chemical shift of partial protons in Figure S20.

fl(ppm)	Ha	Hb	Hc	Hd	He	Hf	H1	H2	H3
Initial δ	3.272	3.669	1.737	1.521	1.323	1.204	6.768	4.259	3.802
Finally δ	3.007	3.178	1.650	1.391	1.215	0.709	6.779	4.302	3.851
$\Delta \delta$	-0.265	-0.491	-0.087	-0.130	-0.108	-0.495	0.011	0.043	0.049

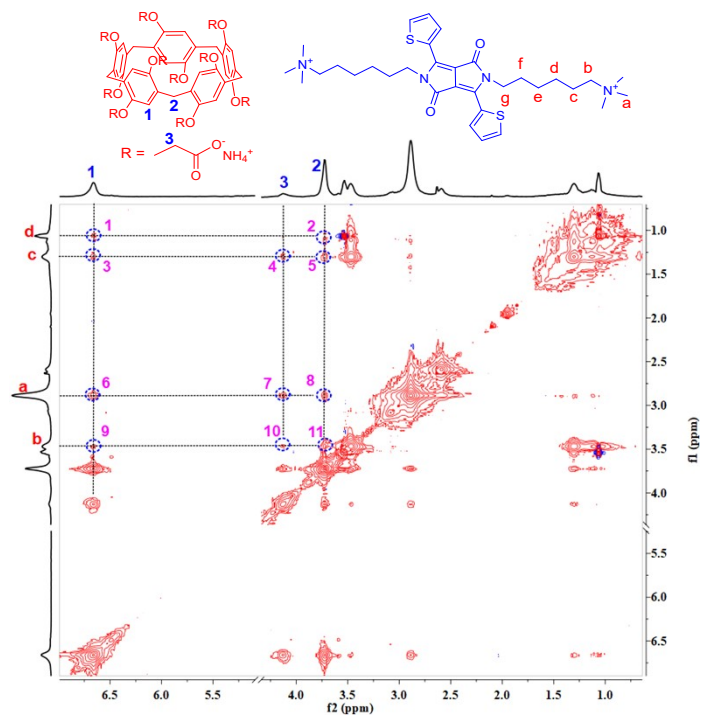


Figure S21. 2D NOESY spectrum of WP5⊃G. [WP5] = 10.0 mM, [G] = 20.0 mM (600 MHz, D₂O, 298 K).

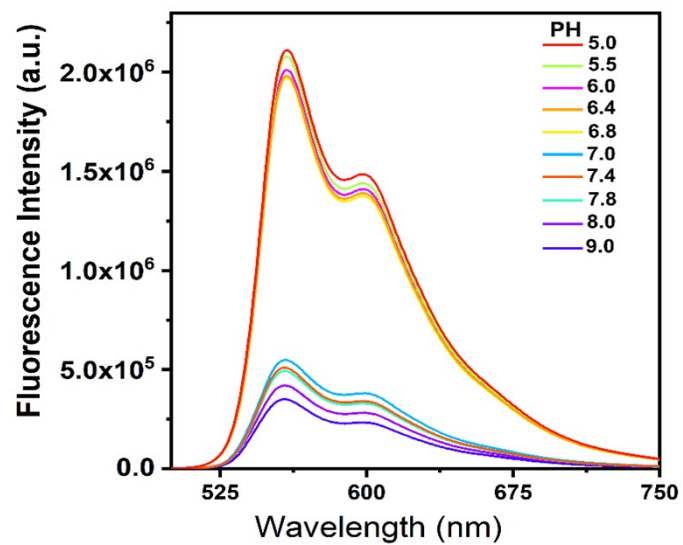


Figure S22. Fluorescence spectral of compound WP5D3G in a different PH solution (phosphate buffer solution), $\lambda_{\text{ex}} = 540$ nm.

6. siRNA loading

The siRNA loading capacity calculation

To determine the siRNA loading capacity of nanoparticles, we first made the calibration curve of the fluorescence intensity versus the concentration of FAM-siRNA. The nanoparticles were treated with in deionized water, after centrifugation (at 10000 rpm for 10 min), the supernatant was measured the fluorescence intensity. The fluorescence intensity of the residual FAM-siRNA was 889 a.u., and the corresponding concentration is calculated to be 0.37 μM through the fitting equation. Thus the DLE and DLC could be calculated as the following formula:

$$\text{Drug loading efficiency (DLE)} = (C_{\text{FAM-siRNA, initial}} - C_{\text{FAM-siRNA, residual}} / C_{\text{FAM-siRNA, initial}}) \times 100\% = 96\%$$

$$\text{Drug loading capacity (DLC)} = m_{\text{FAM-siRNA}} / m_{\text{nanoparticles}}$$

$$\text{DLE} = (10 - 0.37 / 10) \times 100\% = 96\%$$

$$\text{DLC} = 9.63 \mu\text{M} * 13837.67 \text{ g}\cdot\text{mol}^{-1} / 1.73 \text{ g}\cdot\text{L}^{-1} = 7.7 \text{ wt}\%$$

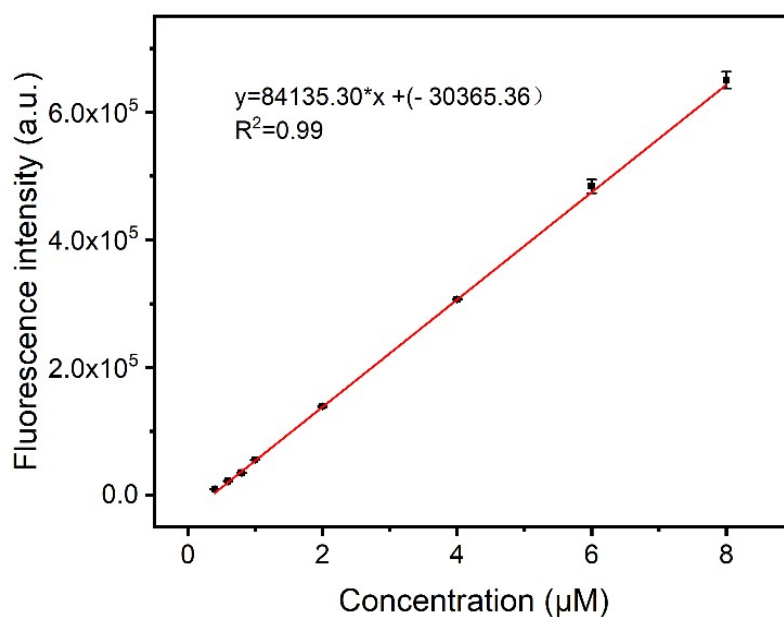


Figure S25. The calibration curve of the fluorescence intensity of FAM-siRNA.

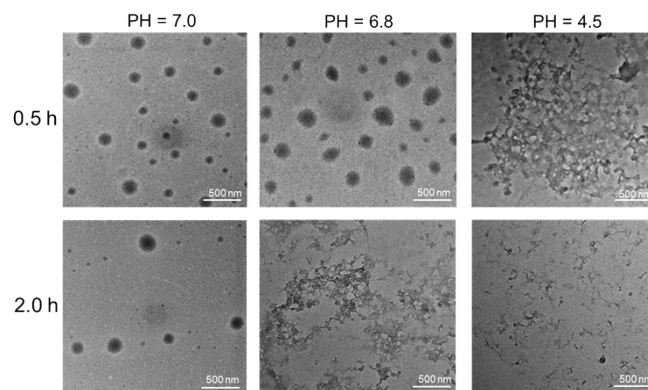


Figure S26. TEM image of WP5-G-siRNA vesicles after incubating for 0.5 h and 2.0 h at different pH conditions.

Data: siRNSAg_NDH		
Model: Sequential Binding Sites		
Chi ² /DoF = 2.729E5		
K1	4.69E6	±5.2E5 M ⁻¹
ΔH1	-7152	±856 cal/mol
ΔS1	-2.61	cal/mol/deg
K2	2.31E5	±6.7E4 M ⁻¹
ΔH2	-4.494E5	±1.21E5 cal/mol
ΔS2	-1.49E3	cal/mol/deg
K3	1.17E5	±2.8E4 M ⁻¹
ΔH3	7.947E5	±1.89E5 cal/mol
ΔS3	2.69E3	cal/mol/deg
K4	4.67E4	±2.2E3 M ⁻¹
ΔH4	-5.023E4	±7.08E3 cal/mol
ΔS4	-1.66E3	cal/mol/deg

Figure S27. Inset data of ITC for Figure 2a.