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Assemble ionic supramolecular polymers using decacationic pillar[5]arene to noncovalently crosslink hyaluronic acid for short DNA delivery

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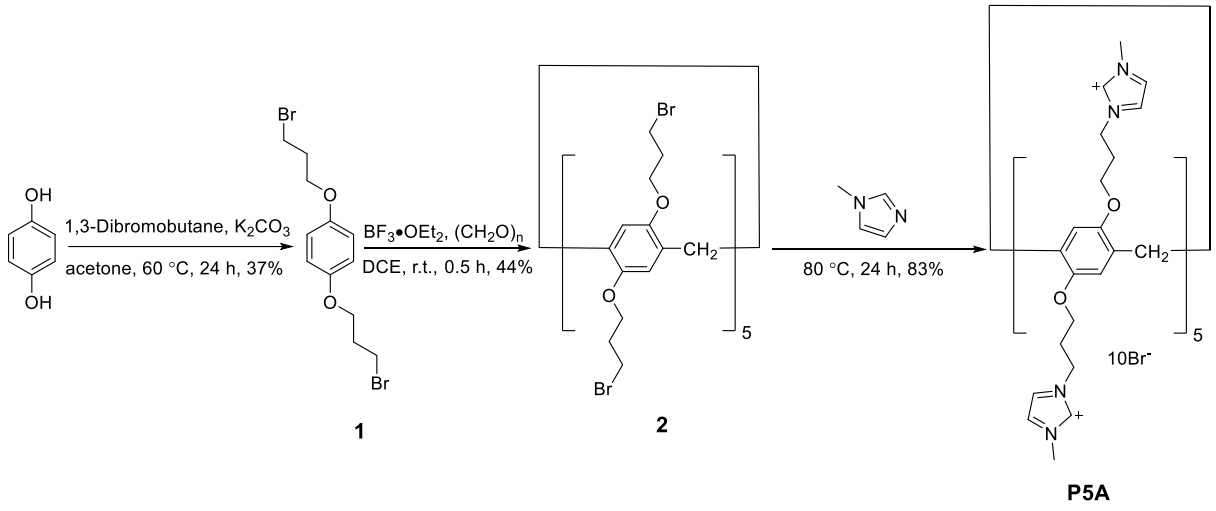
General methods and materials. All the reagents and solvents were commercially available and used as received unless otherwise specified purification. Dynamic light scattering (DLS) experiments were conducted on a Malvern Zetasizer Nano ZS90 using a monochromatic coherent He–Ne laser (633 nm) as the light source and a detector that detected the scattered light at an angle of 90°. Fluorescent measurements were performed on a VARIAN CARY Eclipse Fluorescence Spectrophotometer. Fluorescent microscopic experiments were performed on the Leica DMi8 fluorescent microscope. Flow cytometric experiments were conducted with a Gallios 3L 10C flow cytometry system (Beckman Coulter, USA). Fetal bovine serum (FBS), 1640 Medium, DMEM Medium were purchased from Thermo Fisher Scientific. Cy5-ssDNA-21 and Cy5-dsDNA-21 were commercials purchased from Shanghai HuaGen Biotech Co., Ltd. Lipo2000 was commercial purchased from Thermo Fisher Scientific Co.

Compound P5A. Compound **P5A** was prepared according to the reported method.[1, 2]



Synthesis of 1. A mixture of hydroquinone (8.0 g, 73 mmol), 1,3-dibromopropane (44 g, 0.22 mol), and potassium carbonate (45 g, 0.33 mol) were refluxed in acetone (0.13 L) for 24 h under argon atmosphere. The reaction mixture was cooled to 25 °C and filtered through celite, and the solvent was evaporated under vacuum. The residue was dissolved in dichloromethane (0.1 L), washed with water (2 × 50 mL), 3 N HCl (2 × 50 mL), and brine (2 × 50 mL), dried with sodium sulphate, and concentrated in vacuo. The product was purified by column chromatography (silica gel; eluent: hexane/ethyl acetate). Further purification by recrystallization in ethyl acetate/hexane afforded the title compound as a white solid (9.5 g, 37%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (s, 4H), 4.06 (t, J = 5.8 Hz, 4H), 3.60 (t, J = 6.5 Hz, 4H), 2.33–2.26 (m, 4H).

Synthesis of 2. To a solution of **1** (1.00 g, 2.84 mmol) and paraformaldehyde (0.269 g, 8.97 mmol) in 1, 2-dichloroethane (20 mL), BF_3▪OEt_2 (0.360 mL, 2.86 mmol) was added. The mixture was stirred at 30 °C for 30 min under nitrogen atmosphere. The resulting solution was poured into methanol and the precipitate was collected by filtration. Column chromatography (silica gel; hexane: dichloromethane = 3:2) afforded white solid (454 mg, 0.249 mmol, Yield: 44%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.74 (s, 10H), 3.99 (t, J = 5.8 Hz, 20H), 3.76 (s, 10H), 3.52 (t, J = 6.5 Hz, 20H), 2.26 – 2.20 (m, 20H).

Synthesis of P5A. To a solution of 1-methylimidazole (5 mL), **2** (50.0 mg, 0.0275 mmol) was added. The mixture was heated at 80 °C for 24 h. The resulting solution was poured into diethyl ether and the precipitate was collected by filtration. The reprecipitation process was repeated three times. (**P5A**, 60 mg, 0.0227 mmol, Yield: 83%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.21 (br, 10H), 8.01 (br, 10H), 7.57 (br, 10H), 6.75 (s, 10H), 4.51 (br, 20H), 3.85 – 4.20 (br, 20H), 3.76 (s, 30H), 3.60 (s, 10H), 2.52 – 2.30 (m, 20H).

Cell culture. Hela, MCF-7 and H9C2 cells were obtained from Chinese Academy of Sciences Cell Bank of Type Culture Collection (www.cellbank.org.cn), and incubated in corresponding medium with 10% FBS and 1% penicillin streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Fluorescence microscopy. For microscopic observations, H9C2 cells or Hela cells ($1*10^6$ cells per dish) were seeded in coverglass bottom dishes (35 mm × 35 mm). After culture for 24 hours, the cells were incubated with complete medium containing free Cy5-ssDNA-21, Cy5-ssDNA-21 + **P5A**, Cy5-ssDNA-21 + **ISPs** (Cy5-ssDNA-21 = 2.5 μg/mL; [**P5A**] = 20 μg/mL; [**ISPs**] = 20 μg/mL) at 37 °C for 2 hours. After washing the cells by PBS (1 mL) twice to remove excessive **P5A** or **ISPs** and DNA, the cells were fixed with 4% paraformaldehyde solution, then treated with immunostaining permeabilization Buffer with Triton X-100. Cells were stained with 10 μg/mL Hoechst for 10 min and 75 nM Lyso-tracker Green for 30 min at 37 °C, and then washed with PBS (1 mL) three times. The cells were imaged on Leica DMi8 fluorescent microscope.

In vitro cytotoxicity assay of P5A and ISPs. Take Hela cells for example, the cytotoxicity of **P5A** or **ISPs** against Hela cells was evaluated by the Cell Counting Kit-8 (CCK-8) assay. In brief, Hela cells were seeded in 96-well plates at an appropriate density of 1×10^4 cells per well and then incubated for 24 hours. After adherence, the cells were treated with **P5A** or **ISPs** with different concentrations ranging from 0 to 0.512 mg/mL. After 24 hours of incubation, the medium was replaced with fresh complete medium containing CCK-8. Then, after incubating for 1 hours, the absorbance was measured at 450 nm using a microplate reader (Bio-Tek, Synergy H1, USA). The relative cell viability was calculated as: cell viability = (OD450 (samples)/OD450 (control)) × 100%, where OD450 (control) was obtained in the absence of **P5A** or **ISPs**, and OD450 (samples) was obtained in the presence of **P5A** or **ISPs**. The evaluation with other cells was conducted under similar conditions.

Flow cytometry assay. Hela, MCF-7 and H9C2 cells were seeded at 5×10^5 cells per well in 12-well plate and further cultured for 24 hours. The culture media were replaced by 1 mL of fresh culture medium. Free Cy5-ssDNA-21, Cy5-ssDNA-21 + **P5A** or Cy5-dsDNA-21 + **ISPs** (Cy5-dsDNA-21 = 2.5 μg/mL; [**P5A**] = 20 μg /mL; [**ISPs**] = 20 μg/mL) were added to the cells, respectively. Following incubating for 2 h, the cells were digested, and then followed by flow cytometry assay in FL6 channel on a Gallios 3L 10C flow cytometry system (Beckman Coulter, USA)

Maximum tolerated dose (MTD) evaluations for P5A and ISP-P5A$_{1.0}$HA$_{0.4}$. For the safety profiles of **P5A and ISP-P5A$_{1.0}$HA$_{0.4}$**, 42 ICR mice (18-24 g, n = 6, half female and male) were individually weighed and randomly divided into seven groups. In the control group, mice were i.v. injected within 0.5 mL normal saline. For the another six experimental groups, **P5A**

was i.v. injected with a concentration gradient of 10, 20 and 30 mg/kg, whereas **ISP-P5A$_{1.0}$HA$_{0.4}$** was injected with a concentration gradient of 20, 30 and 40 mg/kg. Body weight was monitored twice a day at defined time and their general behaviors were observed and recorded. After 14 days, the mice were euthanized and organs collected for both control group and high-dose experiment group. The histopathological sections of collected organs were prepared and stained with hematoxylin and eosin (H&E), which was followed by imaging with optical microscopy.

Hemolysis experiment. 5% Human and mouse red blood cells preserved in Alsever's solution were centrifuged in a 1000 r/min centrifuge for 10 minutes to abtain blood samples. Blood samples were diluted with equal volume of isotonic saline. The solution obtained (140 μL) were mixed respectively with saline (560 μL, negative control), deionized water (560 μL, positive control), **P5A**, **ISP-P5A$_{1.0}$HA$_{0.2}$** or **ISP-P5A$_{1.0}$HA$_{0.4}$** solution of different concentrations (2-256 μg/mL). After incubation for 1 h at 37 °C, the samples were centrifugated at 3000 r/min for 10 minutes and the supernatants were obtained. The hemolysis ratio of the samples was evaluated with the absorbance at 545 nm using Allsheng AMR-100 microplate reader.

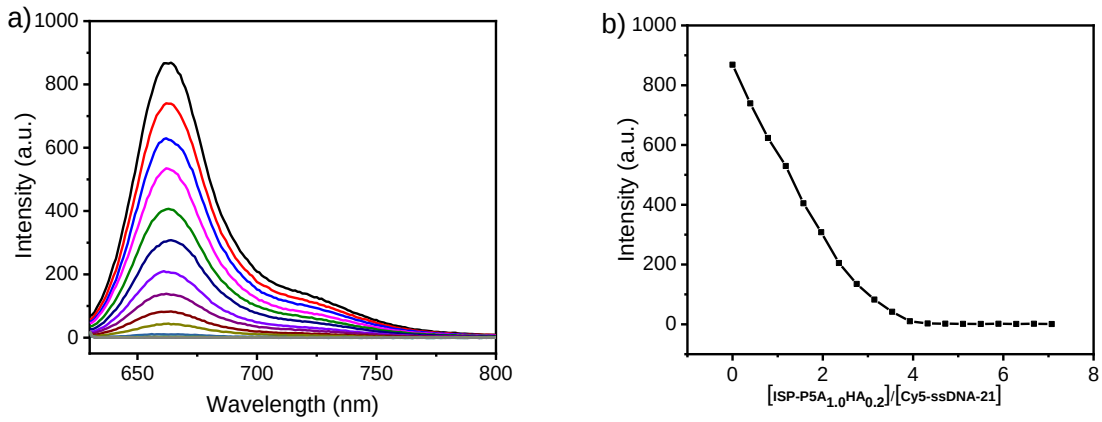


Fig. S1 a) Fluorescence spectra of Cy5-ssDNA-21 (0.5 μM, λ_{ex} = 625 nm) in water with the addition of **ISP-P5A$_{1.0}$HA$_{0.2}$** of incremental amount ([**P5A**] = 0-7.0 equivalents). b) Plot of fluorescence intensity versus [**ISP-P5A$_{1.0}$HA$_{0.2}$**]/[Cy5-ssDNA-21].

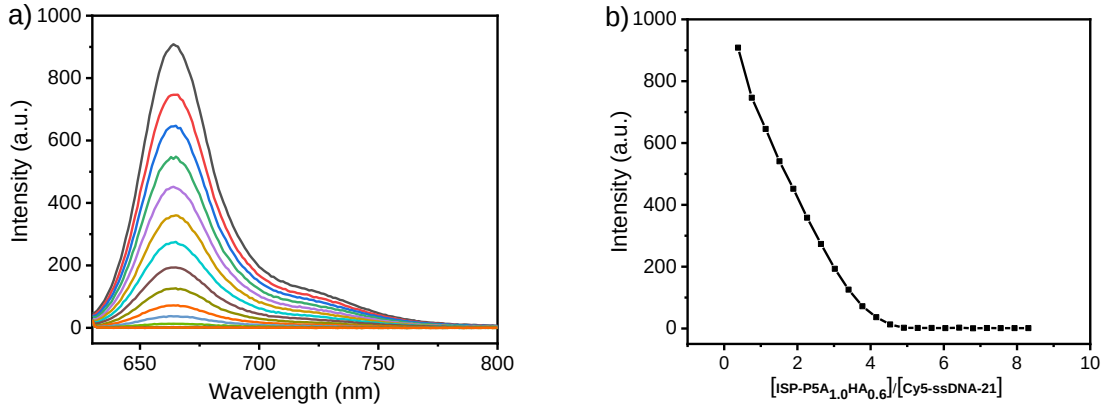


Fig. S2 a) Fluorescence spectra of Cy5-ssDNA-21 (0.5 μM, λ_{ex} = 625 nm) in water with the addition of **ISP-P5A$_{1.0}$HA$_{0.6}$** of incremental amount ([**P5A**] = 0-8.4 equivalents). b) Plot of fluorescence intensity versus [**ISP-P5A$_{1.0}$HA$_{0.6}$**]/[Cy5-ssDNA-21].

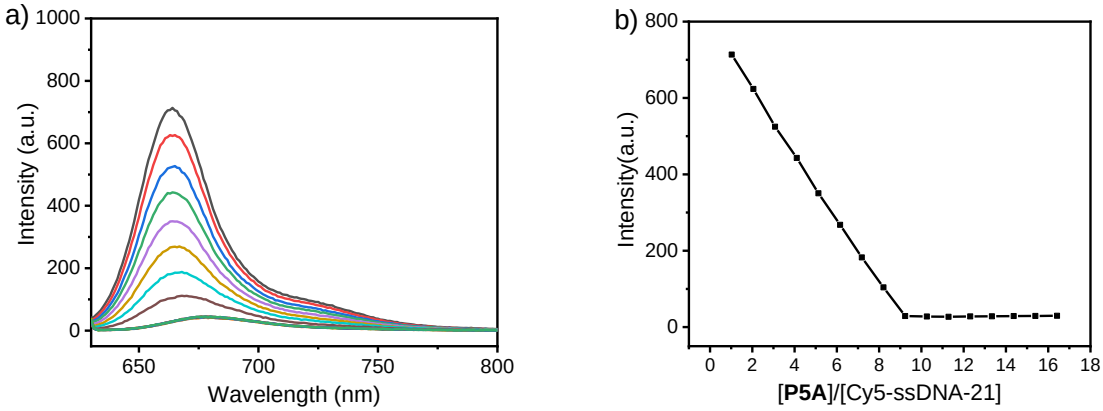


Fig. S3 a) Fluorescence spectra of Cy5-ssDNA-21 (0.5 μM, λ_{ex} = 625 nm) in water with the addition of **P5A** of incremental amount ([**P5A**] = 0-16.5 equivalents). b) Plot of fluorescence intensity versus [**P5A**]/[Cy5-ssDNA-21].

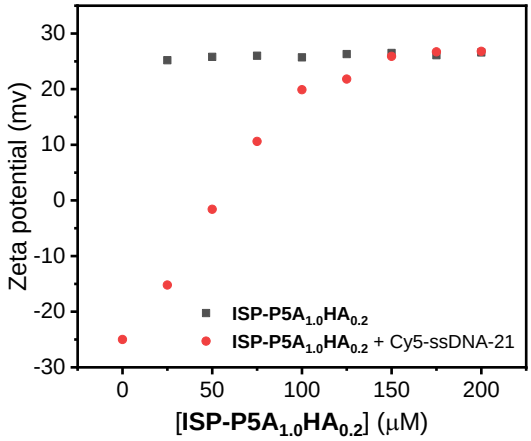


Fig. S4 Zeta potentials of the solutions of ssDNA-21 (10 μM), **ISP-P5A$_{1.0}$HA$_{0.2}$** ([**P5A**] = 25-200 μM) and their mixtures. All the experiments were conducted at 25 °C.

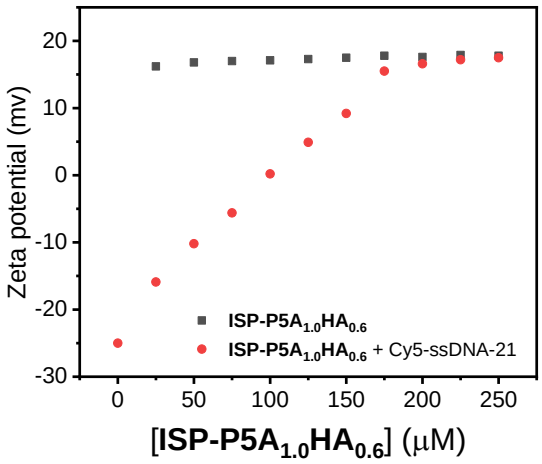


Fig. S5 Zeta potentials of the solutions of ssDNA-21 (10 μM), **ISP-P5A$_{1.0}$HA$_{0.6}$** ([**P5A**] = 25-250 μM) and their mixtures. All the experiments were conducted at 25 °C.

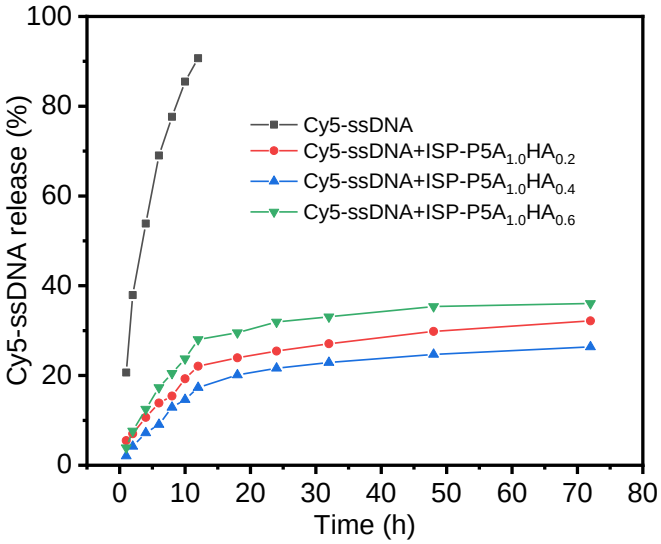


Fig. S6 Dialysis of Cy5-ssDNA (0.5 μM, 0.5 mL) from its solution and the ISP-Cy5-ssDNA solution, molecular weight cutoff = 8 kDa). Amount of Cy5-ssDNA dialyzed was determined using fluorescence spectroscopy.[3]

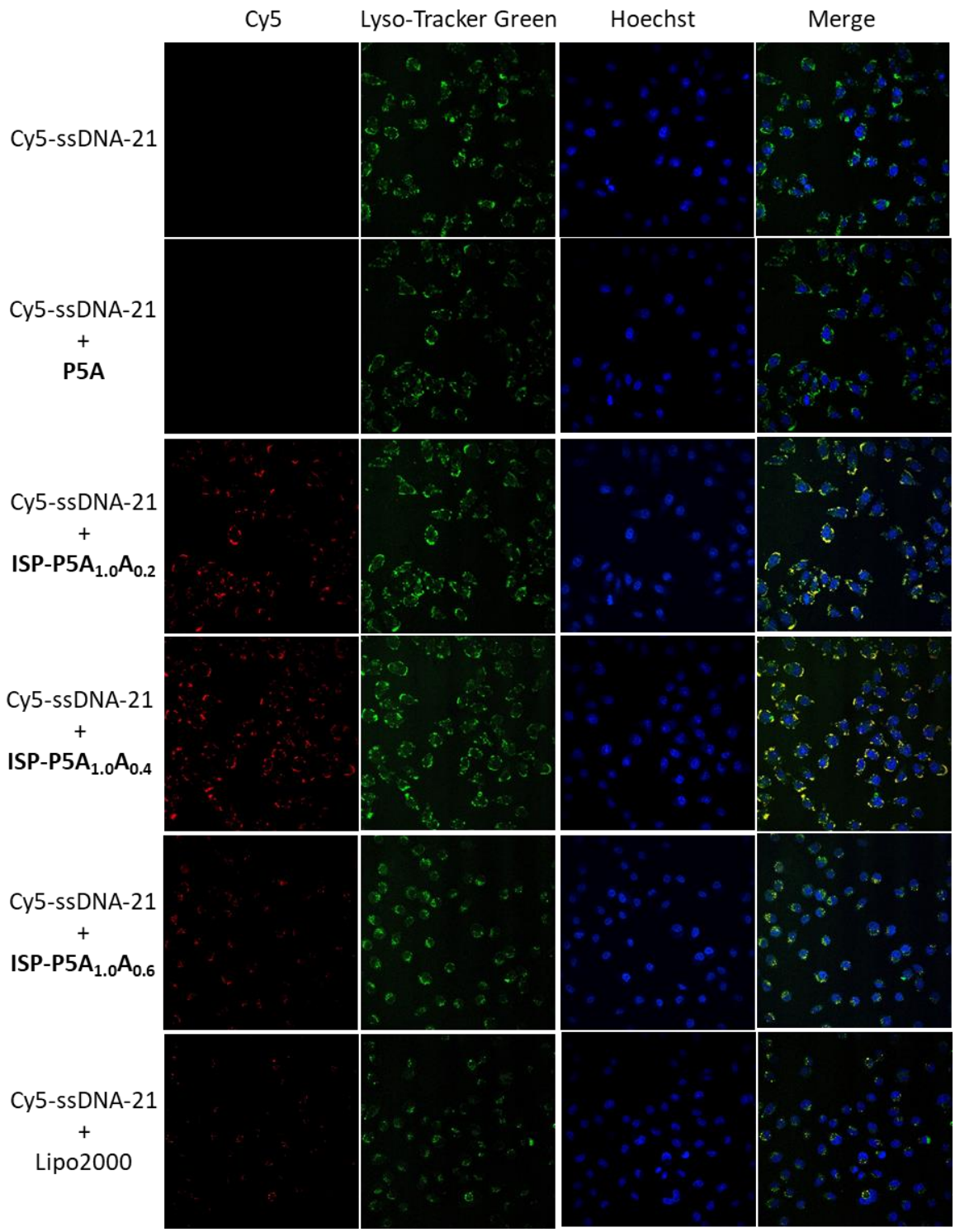


Fig. S7 CLSM images of human Hela cancer cells after incubation for 2 h with Cy5-ssDNA-21, Cy5-ssDNA-21/**P5A**, Cy5-ssDNA-21/**ISP-P5A$_{1.0}$HA$_{0.2}$**, Cy5-ssDNA-21/**ISP-P5A$_{1.0}$HA$_{0.4}$**, Cy5-ssDNA-21/**ISP-P5A$_{1.0}$HA$_{0.6}$** and Cy5-ssDNA-21/Lipo2000. The dosages of Cy5-ssDNA-21, **P5A**, including that of the ISPs and Lipo2000, were 2.5, 20, and 20 μg, respectively. Nuclei and lysosomes were stained with Hoechst (blue) and Lyso-Tracker Green (green), respectively.

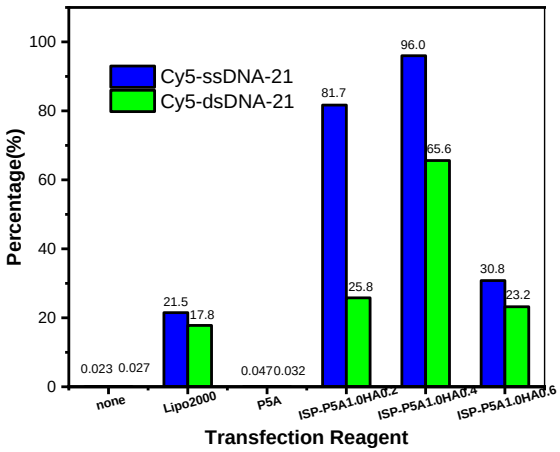


Fig. S8 Delivery (internalization) of Cy5-ssDNA-21 (2.5 μg/mL) and Cy5-dsDNA-21 (5 μg/mL) into Hela cancer cells by none, Lipo2000 (20 μg/mL), **P5A**, **ISP-P5A$_{1.0}$HA$_{0.2}$**, **ISP-P5A$_{1.0}$HA$_{0.4}$** and **ISP-P5A$_{1.0}$HA$_{0.6}$** after incubation for 2 h. For Cy5-ssDNA-21, [**P5A**] = 20 μg/mL for all the latter four cases, and for Cy5-dsDNA-21, [**P5A**] = 40 μg/mL for all the latter four cases.

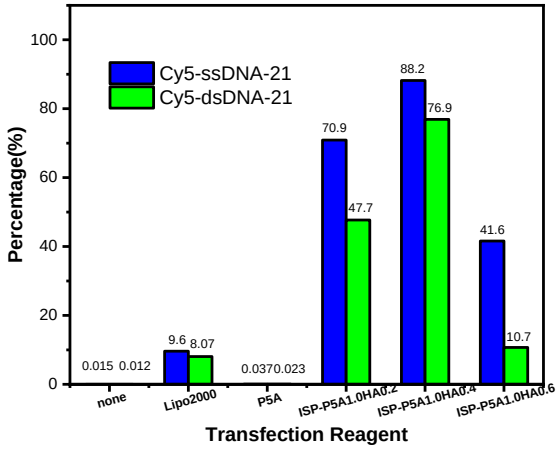


Fig. S9 Delivery (internalization) of Cy5-ssDNA-21 (2.5 μg/mL) and Cy5-dsDNA-21 (5 μg/mL) into MCF-7 cancer cells by none, Lipo2000 (20 μg/mL), **P5A**, **ISP-P5A$_{1.0}$HA$_{0.2}$**, **ISP-P5A$_{1.0}$HA$_{0.4}$** and **ISP-P5A$_{1.0}$HA$_{0.6}$** after incubation for 2 h. For Cy5-ssDNA-21, [**P5A**] = 20 μg/mL for all the latter four cases, and for Cy5-dsDNA-21, [**P5A**] = 40 μg/mL for all the latter four cases.

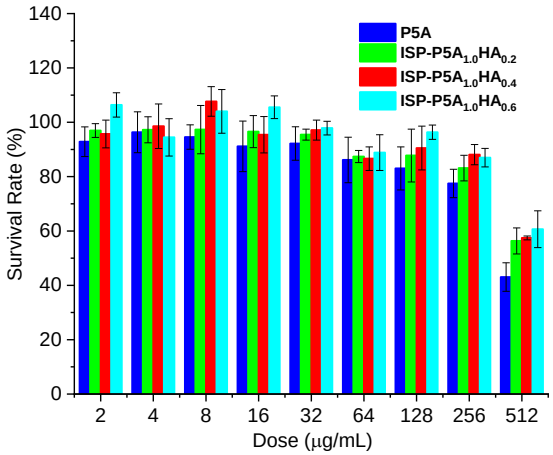


Fig. S10 Cytotoxicity of **P5A**, **ISP-P5A$_{1.0}$HA$_{0.2}$**, **ISP-P5A$_{1.0}$HA$_{0.4}$** and **ISP-P5A$_{1.0}$HA$_{0.6}$** with Hela cancer cells which were incubated for 24 h at 37 °C. The dosage of the ISPs represents that of the **P5A** component. Survival rate are presented as mean ± S.E.M. (n = 3).

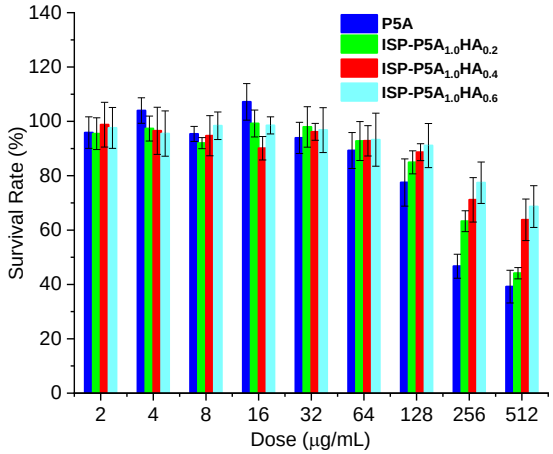


Fig. S11 Cytotoxicity of **P5A**, **ISP-P5A$_{1.0}$HA$_{0.2}$**, **ISP-P5A$_{1.0}$HA$_{0.4}$** and **ISP-P5A$_{1.0}$HA$_{0.6}$** with MCF-7 cancer cells which were incubated for 24 h at 37 °C. The dosage of the ISPs represents that of the **P5A** component. Survival rate are presented as mean ± S.E.M. (n = 3).

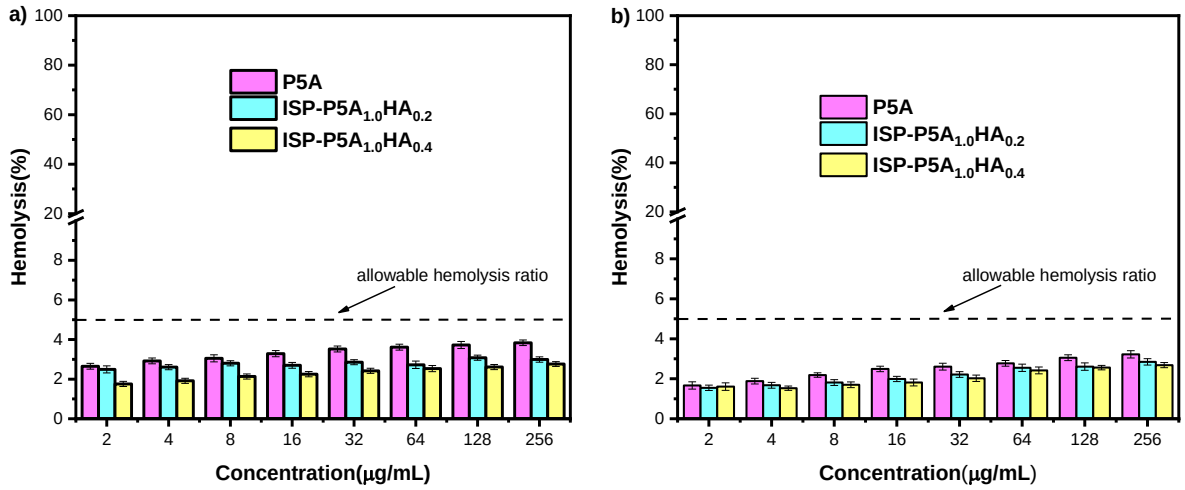


Fig. S12 Hemolytic activity of a) human erythrocytes and b) SD rat erythrocytes treated with **P5A**, **ISP-P5A$_{1.0}$HA$_{0.2}$** and **ISP-P5A$_{1.0}$HA$_{0.4}$** of increasing dosage representing [**P5A**]. Hemolysis data are presented as mean ± S.E.M. (n = 3).

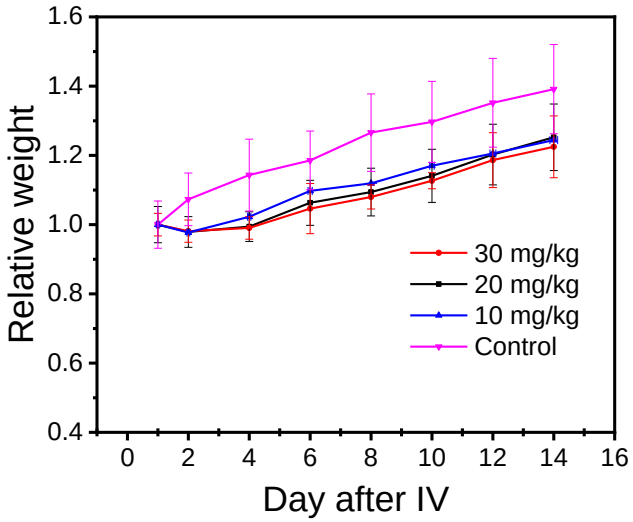


Fig. S13 Weight changes of ICR mice (n = 6, three male and three female) versus time after IV of brine (0.9%) and **P5A** of the indicated doses.

Reference

1. T. Ogoshi, N. Ueshima, T.-a. Yamagishi, Y. Toyota and N. Matsumi, *Chem. Commun.*, 2012, **48**, 3536-3538.
2. Y. Yao, M. Xue, X. Chi, Y. Ma, J. He, Z. Abliz and F. Huang, *Chem. Commun.*, 2012, **48**, 6505-6507.
3. J.-L. Lin, Z.-K. Wang, Z.-Y. Xu, L. Wei, Y.-C. Zhang, H. Wang, D.-W. Zhang, W. Zhou, Y.-B. Zhang, Y. Liu, Z.-T. Li, *J. Am. Chem. Soc.*, 2020, **142**, 3577-3582.