Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Systemic delivery of aptamer-drug conjugates for cancer therapy using enzymatically generated self-assembled DNA nanoparticles

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Experimental Section

Materials

The phosphoramidite building blocks of oligonucleotides were provided by Proligo (CO, USA). 5'-O-DMT-5-fluoro-2'-deoxyuridine-3'-CE phosphoramidite was supplied from Wuhu Huaren Science and Technology (Anhui, China). 3'-Amino-CPG was offered from Glen Research (VA, USA). Cyanine5.5-N-hydroxysuccinimide ester (Cy5.5-NHS ester) was gained from Lumiprobe (MD, USA). Primer and linear template oligonucleotides for rolling circle amplification (RCA) were synthesized by Bioneer (Daejeon, Korea). T4 DNA ligase, its 10X ligation buffer and exonucleases (I and III) were obtained from New England Biolabs (MA, USA). Phi29 polymerase and its reaction buffer were purchased from Lucigen (WI, USA). 5-Fluoro-2'-deoxyuridine-5'-triphosphate (5-F-dUTP) and cyanine 5-6-propargylamino-2'-deoxyuridine-5'-triphosphate (Cy5-dUTP) were acquired from Trilink (CA, USA). Natural dATP, dCTP, dGTP and dTTP were purchased from Promega (WI, USA). Silicon wafer was provided by Ted Pella Inc. (CA, USA). 4', 6-Diamidino-2-phenylindole (DAPI) and SuperSignal™ West Pico PLUS Chemiluminescent substrate were obtained from Thermofisher (MA, USA). DMEM and fetal bovine serum (FBS) were produced by Welgene (Gyeongsangbuk-do, Korea). Annexin V-Cy5 was supplied from Abcam (Cambridge, UK). Antibodies used in western blot analysis were acquired from Cell Signaling Technology (MA, USA). Bradford assay kit was offered by Bio-Rad (CA, USA). Balb/c mice were purchased from Orientbio Inc. (Gyeonggi-do, Korea). Remaining materials were supplied from Sigma-Aldrich (MO, USA).

Synthesis and purification of aptamers

AS1411 and F-AS1411 were synthesized using Mermaid-4 DNA/RNA synthesizer (Bioautomation, TX, USA) following the standard phosphoramidite-based method.[1] Oligonucleotides were cleaved from the CPG resin by deprotection in 33% ammonia solution at 55°C for 16 h. Aptamers were purified using 20% denaturing polyacrylamide gel electrophoresis (PAGE) followed by ethanol precipitation. Concentration of

purified aptamers was determined by measuring UV absorbance at 260 nm. The synthetic yield of aptamers on 1 μmol CPG was 25.2 % for AS1411 and 17.5 % for F-AS1411. AS-1411 and F-AS1411 were characterized using electron spray ionization mass spectrometry (ESI-MS) by Novatia Inc. (PA, USA).

Circular dichroism (CD)

Circular dichroism experiments were conducted at 25°C using Jasco J-1500 CD spectrometer equipped with a Peltier temperature controller (model JFLC-515). Spectra were recorded in 1-mm quartz cuvettes at sample concentration of 10 μM in PBS (containing 5 mM K^+ ion, pH 7.2). The width of spectra was set to 220–340 nm, scan speed was 100 nm/min, 1 nm bandwidth and 0.1 nm spectral resolution.

Thermal difference spectra (TDS)

UV spectra to obtain thermal difference spectra were measured using GENESYS™ 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) by following the previously reported method.[2] The range of measurement was set to 220-335 nm (data interval= 1 nm) at high (90°C) and low (20°C) temperature. Aptamers (3 μM) were prepared in phosphate buffer (1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.2) supplemented with 100 mM KCl. The TDS spectra were obtained by subtracting the spectra recorded at 20°C from the spectra recorded at 90°C.

Preparation of the circular template

Linear template was circularized by ligation reaction. Briefly, 5'-phosphorylated linear template (50 μL, 100 μM) was incubated with primer (75 μL, 100 μM), 10X ligation buffer (1X) and T4 DNA ligase (15 μL, 400 units/μL) at 16°C overnight. Unreacted oligonucleotides were removed by treatment of exonuclease I (3 μL, 20 units/μL) and exonuclease III (6 μL, 100 units/μL) for 1 h at 37°C. The circular template was further purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) followed by ethanol precipitation. The yield of the circular template was calculated to 40.8%.

RCA reaction

The solution containing circular templates (150 μL, 1 μM), natural dATP, dCTP, dGTP, and dTTP (or 5-F-dUTP for NP-F-AS1411) (100 μL, 2 mM), phi29 DNA polymerase (100 μL ,10 units/μL) and its reaction buffer (1X) were incubated for 1 h at 37°C. Then, the reaction mixture was inactivated for 10 min at 65°C. The samples were purified by dialysis in PBS buffer (1X) using 100 KDa amicon filter in order to remove unreacted precursors. The amount of RCA products after the purification was determined to 664.1 μg.

Dynamic light scattering (DLS)

The hydrodynamic size and zeta potential of NP-aptamers (NP-AS1411 and NP-F-AS1411) and NP-Random seq were measured by Zetasizer (Malvern Panalytical Ltd, Malvern, UK). The concentration of the samples used for the DLS analysis was 150 nM.

Scanning electron microscopy (SEM)

NP-AS1411 and NP-F-AS1411 (50 μL, 20 μM) were dried on silicon wafers. Subsequent to coating the samples with Pt, the morphology and size of nanoparticles were observed using Nova Nano SEM 200 (FEI, OR, USA) at an accelerating voltage of 10 kV.

K^+ effect on nano-formulation of NP-AS1411 and NP-F-AS1411

NP-aptamers (50 nM) were incubated in buffers with various concentrations of potassium ion for 1 h at 25°C. The samples were analysed using DLS and agarose gel (1%) electrophoresis.

Cellular uptake imaging

In order to visualize aptamers and NP-aptamers, Cy5.5 was labeled to 3' end of aptamers using Cy5.5-NHS ester and RCA reaction was performed by altering 5% dTTP (or 5-F-dUTP) with Cy5-dUTP. CT26 cells (murine colorectal carcinoma cell line) were seeded on a 35-mm glass-bottom dish (1×10^5 cells/dish) and

incubated for 24 h in DMEM containing 10% FBS and 1% penicillin /streptomycin. After the media was replaced to serum-free DMEM, the cells were treated with Cy5.5-labeled aptamer (2 μM) or Cy5-labeled NP-aptamers (10 nM) for 6 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. At the end of the treatment, they were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Subsequent to washing with PBS, nuclei were stained with DAPI for 10 min. Confocal laser scanning microscopy (CLSM; LSM700, Carl Zeiss Microscopy, Oberkochen, Germany) was used to observe aptamers and NP-aptamers in the cells at the magnitude of ×400.

Flow cytometry

CT26 cells and non-cancer cell lines (MLg cells, NIH/3T3 cells, and bEnd.3 cells) (1×10^5 cells) were harvested and suspended in serum-free medium. They were incubated with Cy5.5-labeled aptamers (2 μM) or Cy5-labeled nanoparticles (NP-aptamers or NP-Random seq) (10 nM) for 6 h at 37°C in a rotator. Cell suspensions were centrifuged for 3 min at 1500 x g and washed twice with ice-cold PBS. Cell pellets were resuspended in 1 mL of ice-cold PBS and fluorescence intensity of the cells was determined by flow cytometry (Guava, Millipore, MA, USA). Triplicated samples of at least 10,000 events were analysed.

Cellular uptake mechanistic study

CT26 cells (1×10^5 cells) were pre-incubated with various endocytosis inhibitors (chlorpromazine (CPZ; 10 μM), methyl-β-cyclodextrin (MβCD; 1 mM), ethyl isopropyl amiloride (EIPA; 50 μM), and incubation at 4°C) in serum-free DMEM for 30 min before treatment of Cy5.5-labeled aptamers (2 μM) or Cy5-labeled NP-aptamers (10 nM). The samples for flow cytometry analysis were prepared as described in flow cytometry section. The uptake levels of aptamers (or NP-aptamers) in the presence of endocytosis inhibitors were normalized to those of aptamers (or NP-aptamers) in the absence of the inhibitors. The data were obtained by performing experiments in triplicate.

Serum stability

Serum stability of aptamers (3 μM) and NP-aptamers (15 nM) were evaluated by incubating with 50% mouse serum at 37°C for pre-determined time points (0, 1, 2, 3, 4, 5, 6, 12, and 24 h). At the end of each time point, the samples were treated with 80 μg of proteinase K, 50 μM EDTA, and 0.1% sodium dodecyl sulfate (SDS) for 1 h at 37°C to degrade serum proteins. The samples were analysed with 20% denaturing PAGE for aptamers and 1% agarose gel electrophoresis for NP-aptamers using an iBright™ FL1000 (Invitrogen, CA, USA). Remaining aptamers and NP-aptamers were quantified by densitometry of the gel band with Image J software 1.45 (National Institutes of Health, MD, USA). The data were obtained by performing experiments in triplicate.

Nuclease digestion assay.

NP-F-AS1411 (50 nM) was incubated with DNase I (0.1 units/mL) or mixture of exonuclease I (0.1 units/mL) and exonuclease III (0.2 units/mL) in acetate buffer (pH 5) or phosphate buffer (pH 7) at 37°C for various time points. The samples were then heated at 80°C for 20 min to inactivate the enzyme and analysed using agarose gel (1%) electrophoresis.

Cell viability assay

CT26 cells were seeded in 96-well culture plate (5×10^3 cells/well) and incubated at 37°C in humidified atmosphere containing 5% CO_2. When the confluency of cells was approximately 80%, the cells were washed with PBS and incubated with fluorouracil (FUdR), aptamers or NP-aptamers at various concentrations in serum-free DMEM for 6 h at 37°C. The cells were washed with PBS, exchanged with serum-containing DMEM and further incubated for 72 h at 37°C in a 5% CO_2 incubator. The cells were washed with PBS and treated with 10 μL CCK-8 solution in DMEM (100 μL) for 4 h. The cell viability was calculated from the absorbance measured at 450 nm using a microplate reader (Molecular Devices, CA, USA). The data were obtained by performing triplicated experiments.

Tumour-bearing mouse model

All experiments with live animals were performed in compliance with the relevant laws and institutional guidelines of KIST, and the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Science and Technology (KIST) (the committee chair: Dr. Heh-In Im, the head of Research Animal Resource Center, KIST) have approved the experiments (2020-005). CT26 cells (2×10^6 cells/mouse) were subcutaneously injected to flank of male Balb/c mice. The tumour was allowed to grow for a mean volume of 80 mm^3 for *in vivo* fluorescence imaging experiments and antitumour studies.

Biodistribution

PBS, Cy5.5-labeled aptamers (9 μM, 200 μL) or Cy5-labeled NP-aptamers (450 nM, 200 μL) was administrated via intravenous (IV) route. At 1, 4, 8, 12, 16, and 24 h post-injection, the mice were anesthetized and fluorescence images were taken with IVIS spectrum *in vivo* imaging system (PerkinElmer, MA, USA). For *ex vivo* imaging, the mice were sacrificed to harvest major organs (brain, heart, lung, liver, spleen, kidney, and tumour) at 12 h post-injection.

***In vivo* antitumour efficacy study**

The CT26 tumour-bearing mice were randomly divided into four groups (6 mice/group). PBS, FUdR, NP-AS1411 or NP-F-AS1411 at equivalent FUdR concentration (a dose of 5 mg/kg body weight, 200 μL) was intravenously administrated via tail vein in every 3 days for 30 day-period. The control group only received an equal amount of PBS injection. The tumour volume was measured by using the digital caliper in every 3 days and calculated according to the formula: $V\ (mm^3) = 1/2 \times L\ (mm) \times W\ (mm) \times W\ (mm)$, where L is the tumour length and W is the tumour width. The body weight of mice was also recorded using an electronic balance at the time of each treatment. Mice were sacrificed on day 30, final tumour weight was recorded, and major organs were harvested for apoptosis analysis.

Apoptosis assay

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin and cut to 4-µm-thickness to mount them on glass slides. Sections were deparaffinized by heating the slides for 1 h followed by 10 min incubation in a xylene bath at room temperature. To rehydrate the sections, they were treated with ethanolic solution (100%, 95%, 85%, and 75%) and washed with PBS. Then, the sections were immersed in a jar filled with boiling citrate buffer (pH 6) at 95°C for 20 min. After cooling down the sections at room temperature for 20 min, they were washed with PBS and endogenous peroxidase was inactivated by covering them with 3% hydrogen peroxide for 15 min at room temperature, followed by washing with PBS for 10 min. Annexin V-Cy5 was employed to stain the sections in a humidified chamber overnight at 4°C. Nuclei of tissues were stained with DAPI for 5 min at room temperature. The sections were applied to the cover slip and visualized under CLSM (LSM 700) at the magnitude of ×400.

Western blot

Harvested tissues were frozen in liquid nitrogen and homogenized with ice-cold RIPA buffer ((Tris-HCl (pH 7.5; 50 mM), sodium chloride (150 mM), Nonidet P-40 (1.0 %), sodium deoxycholate (0.5%), and SDS (0.1%)) supplemented with NaF (1 mM) and protease inhibitor cocktails (1 tablet). Tissue lysates were transferred to 2 mL tube and maintained under agitation for 16 h at 4°C. The lysates then were centrifuged for 20 min at 2500 x g and the supernatants were used for western blot analysis. After quantifying total protein concentration by using Bradford assay kit, equal amount of protein (10 µg/lane) was loaded to SDS-PAGE and then electrophoretically transferred to a poly-vinylidene difluoride (PVDF) membrane. The membranes were incubated in TBST and 5% bovine serum albumin to block non-specific binding. Then membranes were incubated overnight with thymidylate synthase (1:1000) or p53 (1:1000). β-actin (1:1000) was used as a loading control. The membranes were further incubated for 1 h at room temperature with HRP-linked anti-rabbit IgG (1:2000). After washing with TBST, the membranes were treated with SuperSignal™ West Pico PLUS Chemiluminescent substrate for 3 min and observed using iBright FL1000. Image J software was employed to quantify protein band intensity.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed using Origin 9.0 (Originlab, MA, USA). Tukey's multiple comparison test was performed to assess the statistical significance of the differences among samples.

Table S1. The sequences aptamers, the primer, and the templates used for rolling circle amplification. F denotes FUdR monophosphate (the primer biding sequence and the sequence complementary to the aptamer are indicated in blue and red, respectively).

			Sequence (5' to 3')
AS1411			GGTGGTGGTGGTTGTGGTGGTGGTGG
F-AS1411			GGFGGFGGFGGFFGFGGFGGFGGFGG
RCA	**Primer**		CTCTGGTGAGGACAGGACTT
	Linear template	**For NP-AS1411 and NP-F-AS1411**	Phosphate-CTCACCAGAGCCACCACCACCACAACCACCACCACCAAAAAAACCACCACCACCACAACCACCACCACCAAGTCCTGTC
		For NP-Random seq	Phosphate-CTCACCAGAGACATGTTAGTCGTATCGAGTGATAGTAAAAAAAACATGTTAGTCGTATCGAGTGATAGTAAGTCCTGTC

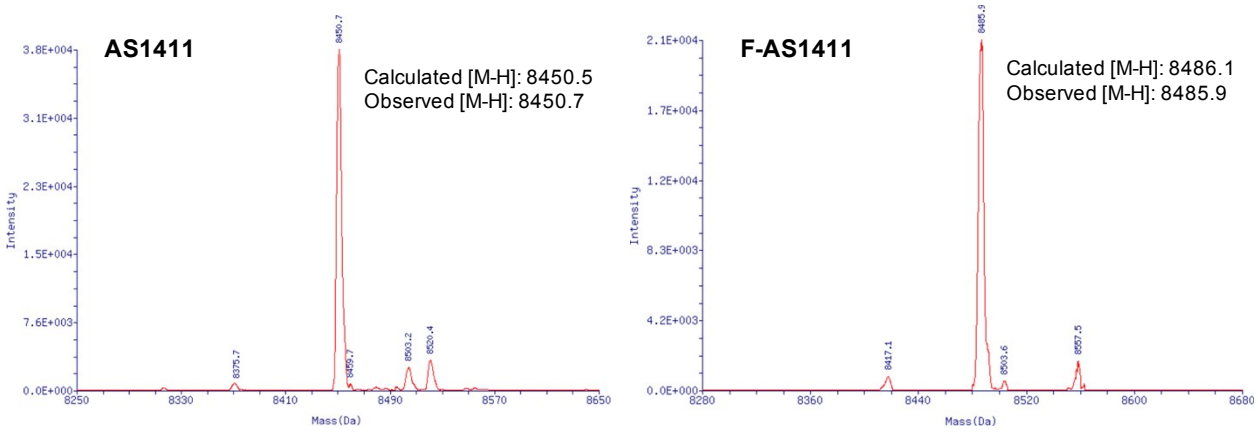
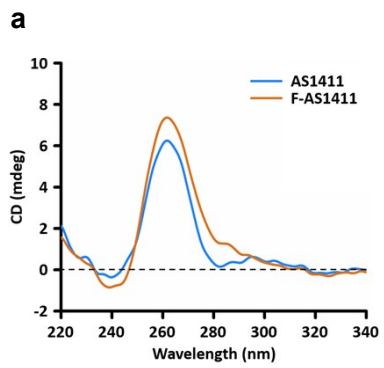
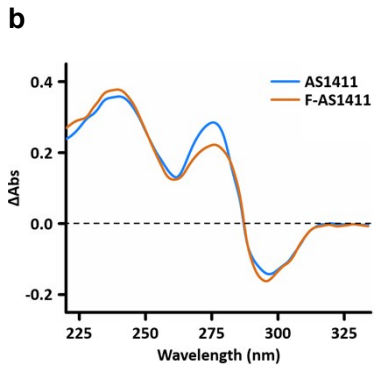


Fig. S1. Electrospray ionization mass spectrometry (ESI-MS) of AS1411 (left) and F-AS1411 (right).





	TDS factor		
	$\frac{\Delta A240}{\Delta A295}$	$\frac{\Delta A255}{\Delta A295}$	$\frac{\Delta A275}{\Delta A295}$
AS1411	2.47	1.28	1.93
F-AS1411	2.28	1.05	1.33
Antiparallel	< 2	< 1.5	< 2
Parallel	> 4	> 3.5	> 3

Fig. S2. (a) Circular dichroism (CD) spectra of AS1411 and F-AS1411 (10 μM). (b) Thermal difference spectra (TDS) of AS1411 and F-AS1411 (3 μM).

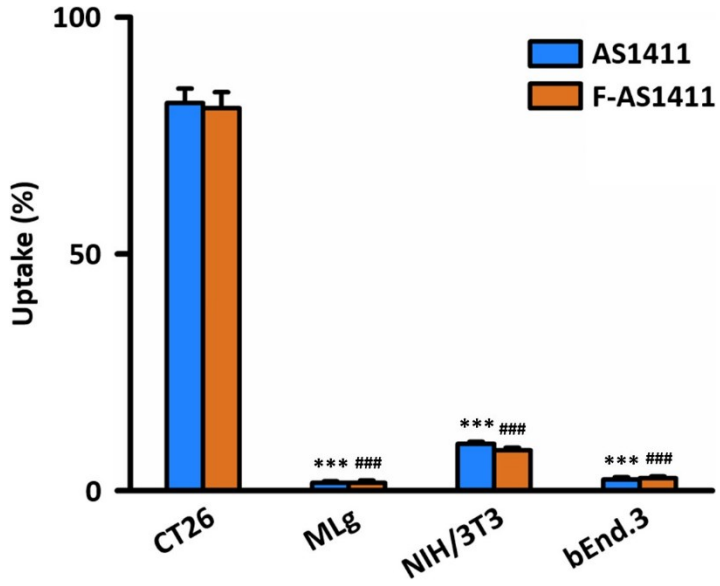


Fig. S3. Cellular uptake efficiency of AS1411 and F-AS1411 in cancer (CT26) and non-cancer (MLg, NIH-3T3, and bEnd.3) cell lines. $^{***}P<0.001$, $^{###}P<0.001$ *vs.* CT26.

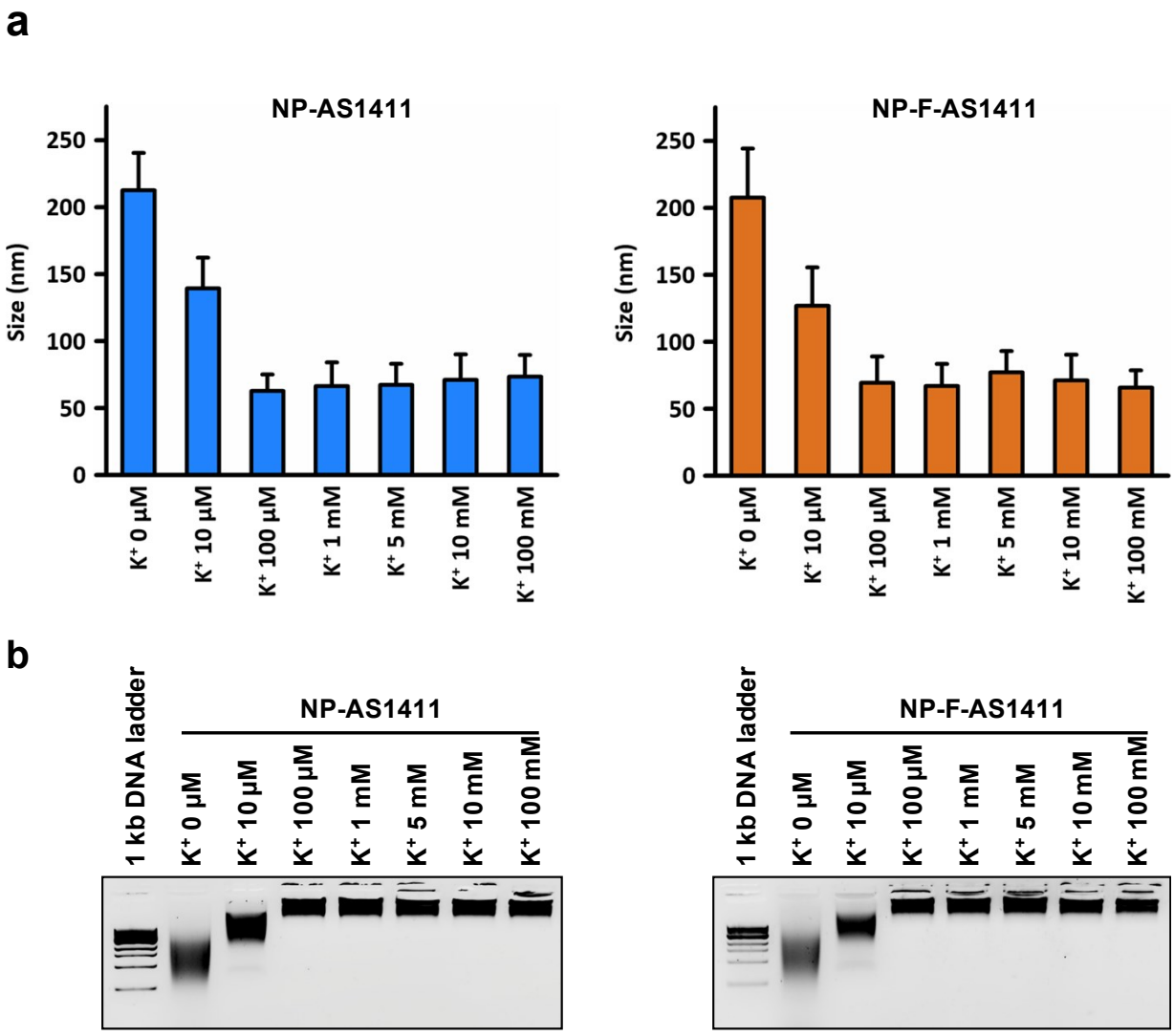


Fig. S4. (a) Hydrodynamic sizes of NP-AS1411 and NP-F-AS1411 produced at varying concentrations of potassium. (b) Agarose gel (1%) analysis of NP-AS1411 and NP-F-AS1411 produced at varying concentrations of potassium.

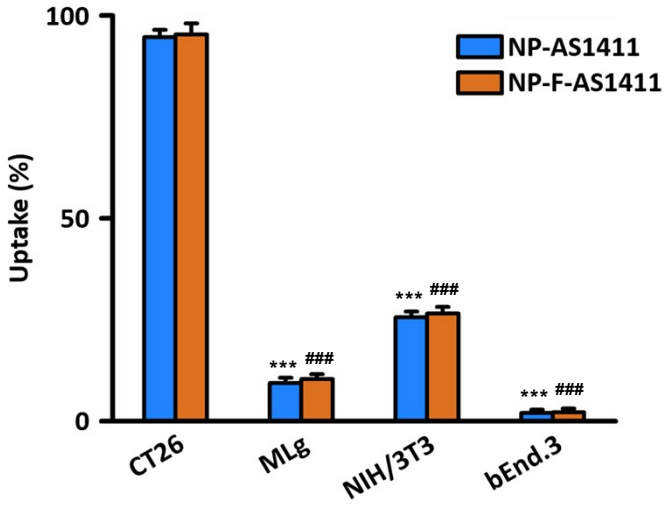


Fig. S5. Cellular uptake efficiency of NP-AS1411 and NP-F-AS1411 in cancer (CT26) and non-cancer (MLg, NIH-3T3, and bEnd.3) cell lines. $^{***}P<0.001$, $^{###}P<0.001$ *vs.* CT26.

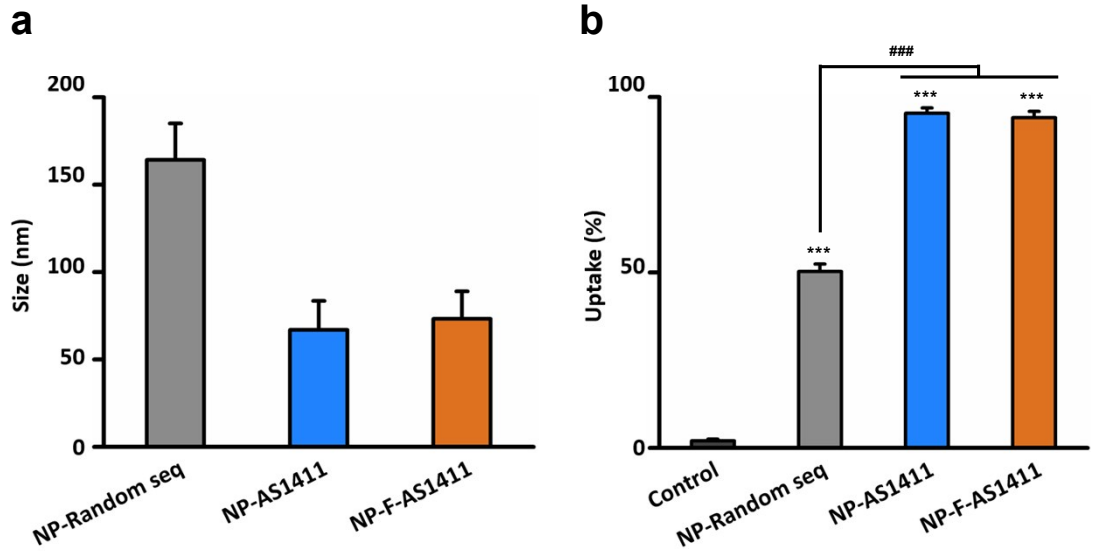


Fig. S6. (a) Hydrodynamic sizes of nanoparticles produced by RCA. (b) Cellular uptake efficiencies (CT26) of nanoparticles produced by RCA. $^{***}P<0.001$ *vs*. Control; $^{###}P<0.001$.

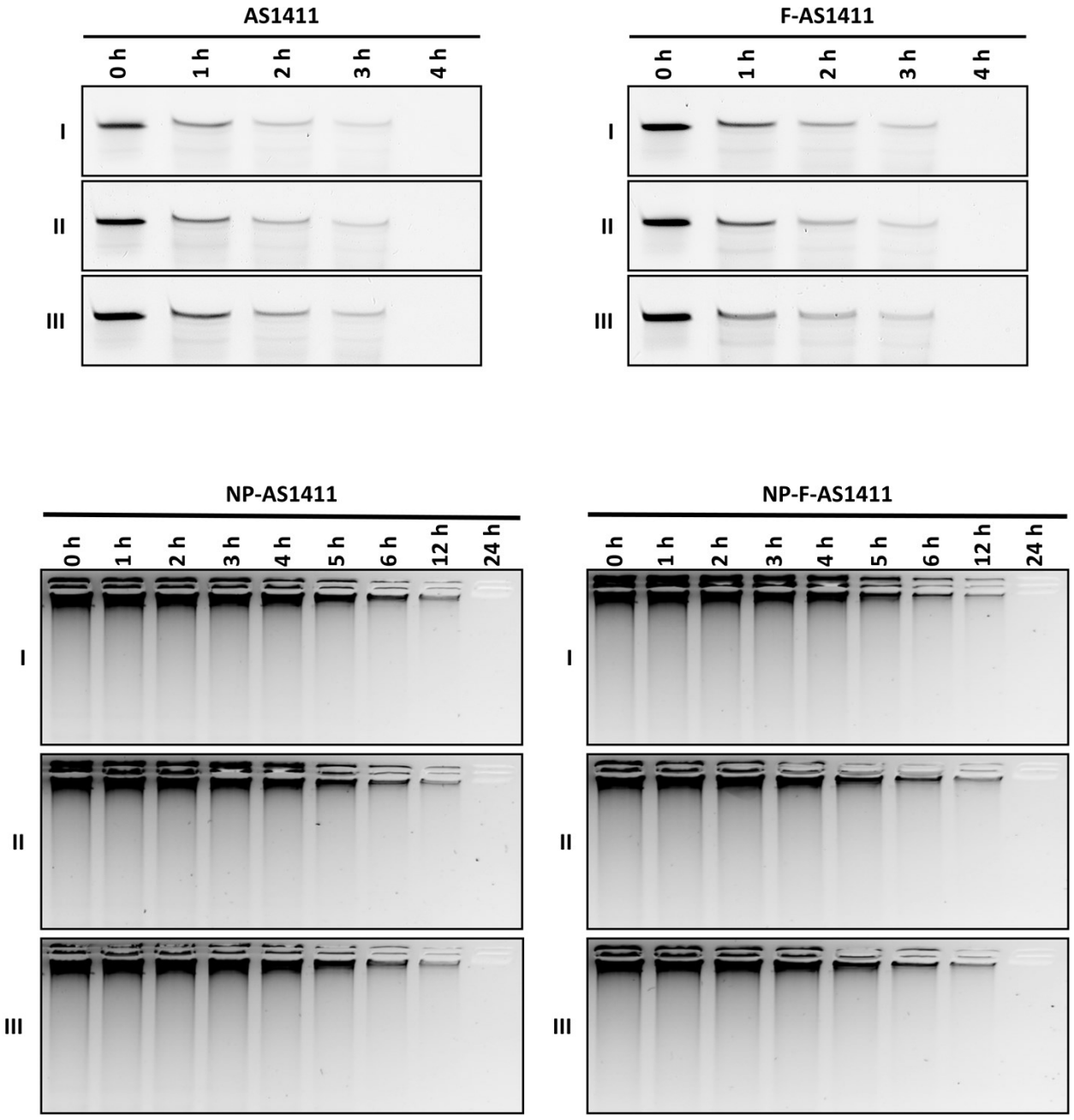


Fig. S7. Denaturing PAGE (20%) analysis of AS1411 and F-AS1411 (top) and agarose gel (1%) analysis of NP-AS1411 and NP-F-AS1411 (bottom) after incubation in 50% mouse serum for 0, 1, 2, 3, 4, 5, 6, 12, 24 h. Three independent experiments were performed for each material.

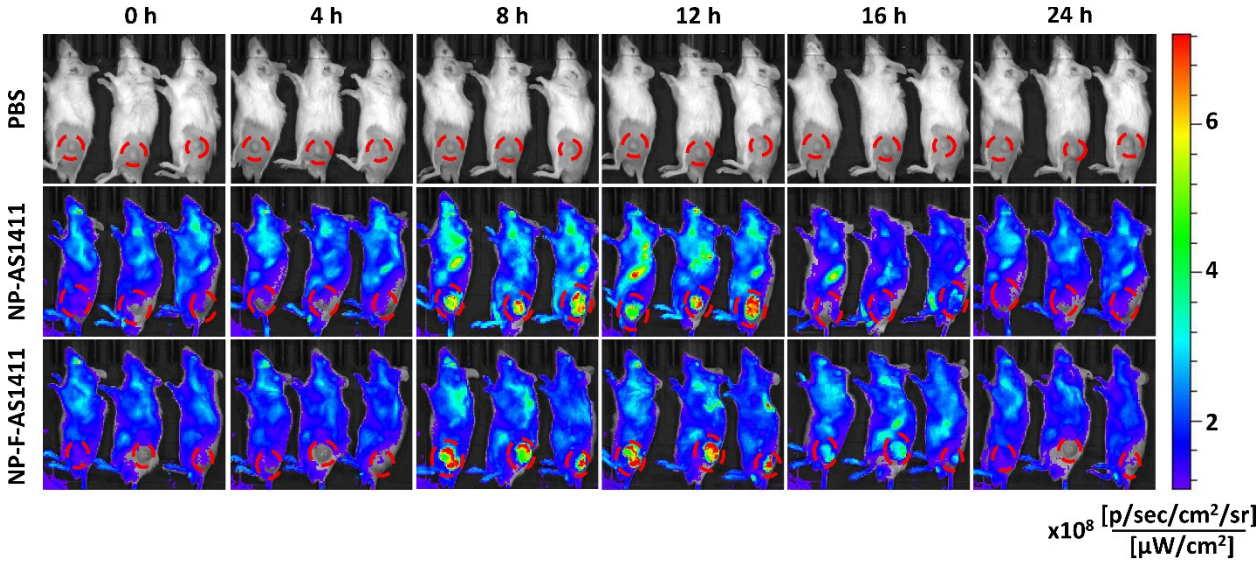


Fig. S8. *In vivo* biodistribution images of intravenously injected NP-AS1411 and NP-F-AS1411 in CT26 tumour-bearing mice (n=3). The tumour region was indicated by red circle.

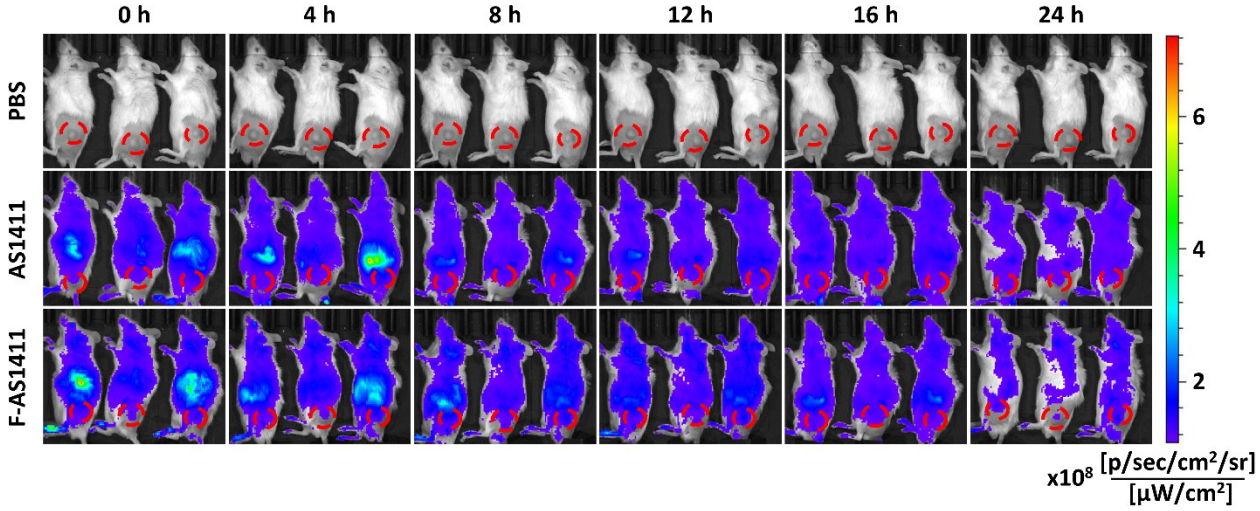


Fig. S9. *In vivo* biodistribution images of intravenously injected AS1411 and F-AS1411 in CT26 tumour-bearing mice (n=3). The tumour region was indicated by red circle.

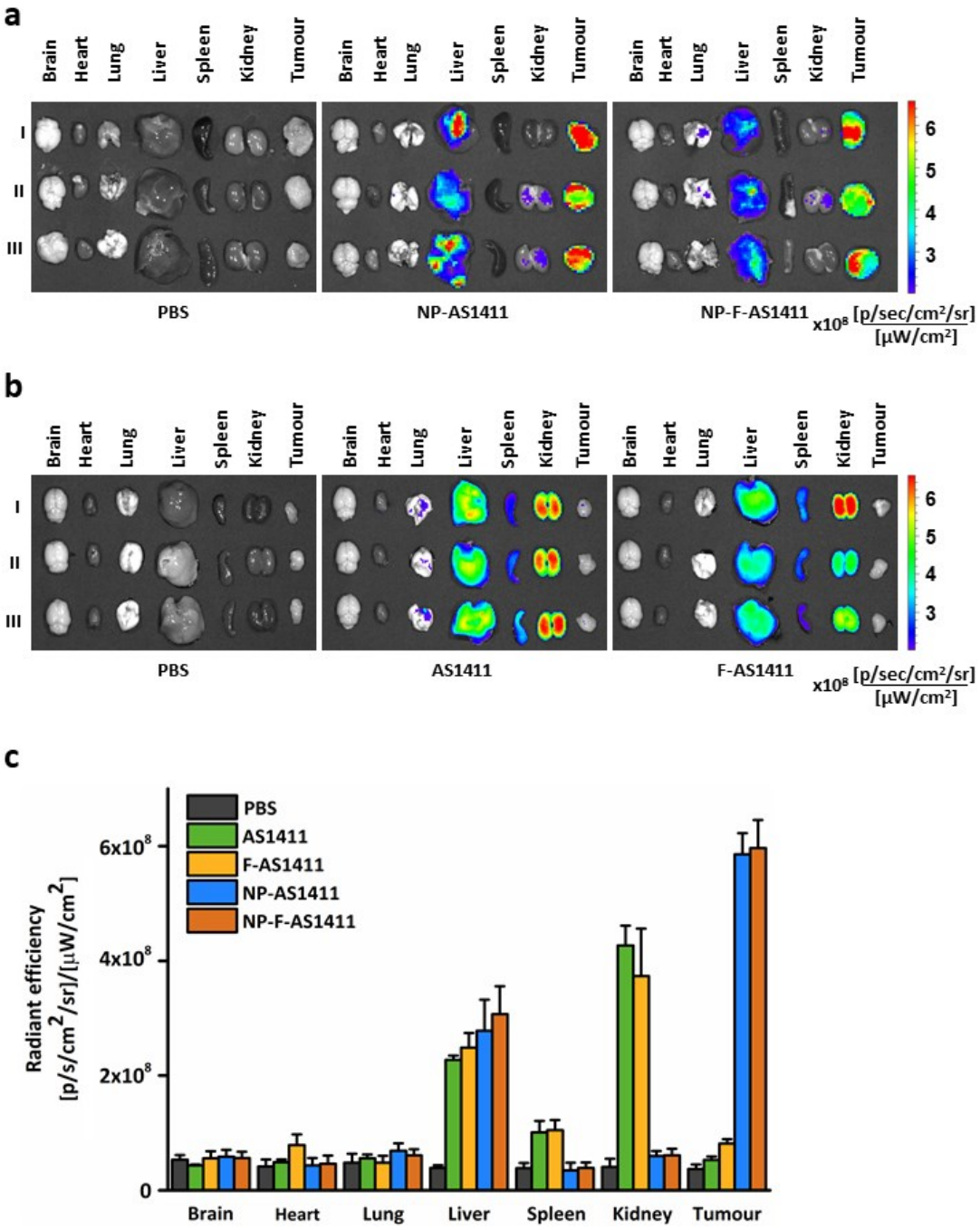


Fig. S10. (a) *Ex vivo* images of major organs harvested from mice 12 h after intravenous injection of PBS, NP-AS1411, and NP-F-AS1411 (n=3). (b) *Ex vivo* images of major organs harvested from mice 12 h after intravenous injection of PBS, AS1411, and F-AS1411 (n=3). (c) Quantitative analysis of fluorescence intensity measured from *ex vivo* images.

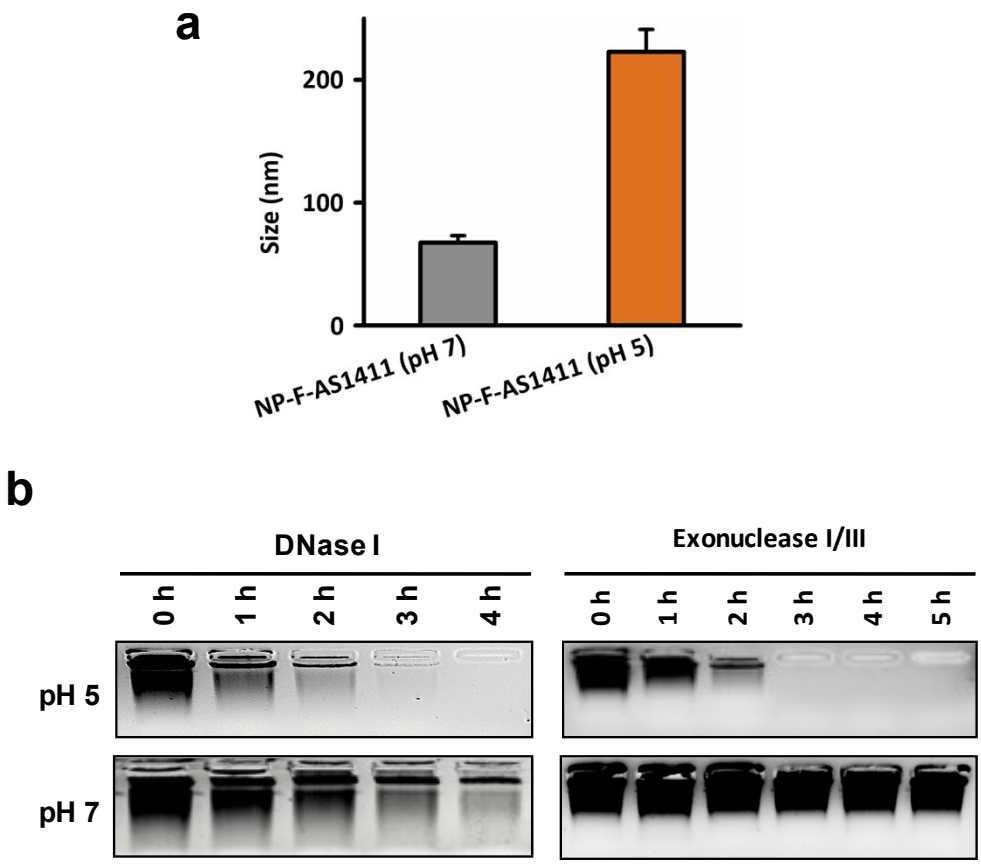


Fig. S11. (a) Hydrodynamic sizes of NP-F-AS1411 at pH 5 and 7. (b) Agarose gel (1%) analysis of NP-F-AS1411 after treatment with DNase I (left) and exonuclease I/III (right) at pH 5 and 7.

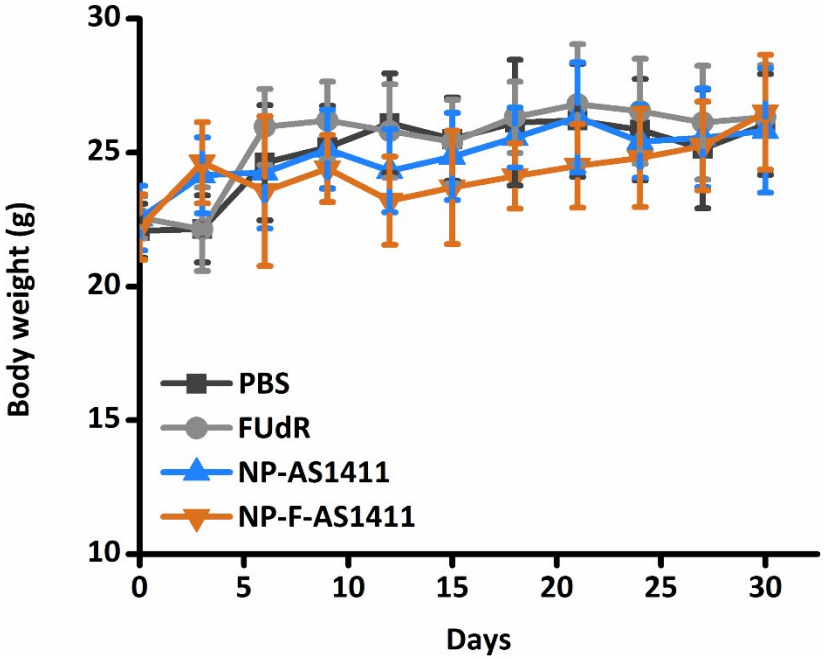


Fig. S12. The body weight change of mice during the treatment period (n=6).

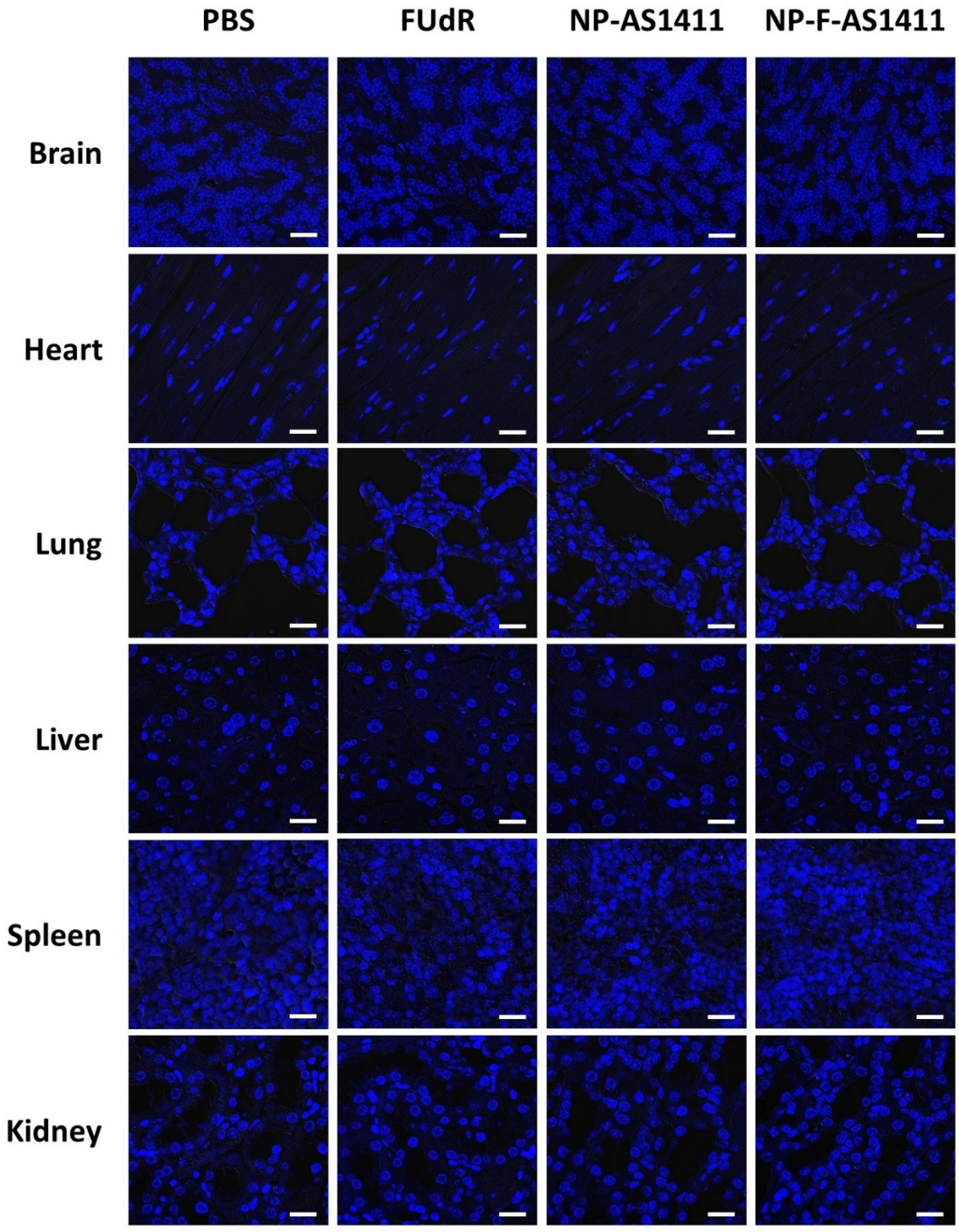


Fig. S13. Confocal microscopic images of sections of major organs. Apoptotic cells and nuclei were stained with annexin V-Cy5 (red) and DAPI (blue), respectively. Scale bar indicates 20 μm.

Reference

1. S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, **48**, 2223-2311.

2. J. Carvalho, A. Paiva, M. P. C. Campello, A. Paulo, J.-L. Mergny, G. F. Salgado, J. A. Queiroz and C. Cruz, *Sci. Rep*., 2019, **9**, 1-12.