Supplementary information

ATP stabilised and sensitised calcium phosphate nanoparticles as effective adjuvants for DNA vaccine against cancer

Bing Sun[1], Xiaohui Zhao[1,2], Wenxi Gu[1,3], Pei Cao[1], Fatemeh Movahedi[1], Yanheng Wu[1,4], Zhi Ping Xu[1] ✉, Wenyi Gu[1] ✉

1. *Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St Lucia, QLD 4072, Australia*
2. *GMU-GIBH Joint School of Life Sciences, Guangzhou Medical University, Guangzhou 511436, China.*
3. *Institute of Veterinary Medicine, Xinjiang Academy of Animal Science, Urumqi, 830011, China.*
4. *Gillion ITM Research Institute, Guangzhou Hongkeyuan, Guangzhou 510530, China.*

✉ **Corresponding authors:**

Address: Australian Institute for Bioengineering and Nanotechnology, Building 75, Corner of Cooper Road & College Road, University of Queensland, St Lucia, Qld 4072, Australia

ZX: email: gordonxu@uq.edu.au, Tel: 61-7-334 63809; WG: email: w.gu@uq.edu.au, Tel: 61-7-33463814

Experimental

Materials

Chemicals The fetal bovine serum (FBS) was purchased from Corning Inc. Bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640 Medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI) dye, TMB Chromogen Solution, Lipofectamine 2000, 6× loading buffer, penicillin/streptomycin and ATP (adenosine 5'-triphosphate) were purchased from Thermal Fisher SCIENTIFIC. Phosphate buffered saline (10 × PBS) were purchased from Lonza. LysoSensor™ Green DND-189 and TRIzol reagent were purchased from Life Technologies. NucleoBond® Xtra Maxi was acquired from Macherey-Nagel. CellTiter-Glo® Luminescent Cell Viability (ATP) Assay kit was purchased from Promega. Cy5-dsDNA was purchased from Sigma-Aldrich. High Capacity cDNA Reverse Transcription Kit and PowerUp SYBR Green Master Mix were purchased from Applied biosystems. Other chemicals and reagents were from Sigma-Aldrich if not described specifically. Water (H_2O) used in experiments was deionised Milli-Q water. All the other chemicals were of analytical or reagent grade.

Animals Mice were acquired from the University of Queensland Biological Resources and kept in filter-topped cages with standard rodent chow and water in a 12 h light/dark cycle. Female C57BL/6J mice were used for vaccination and tumour inoculation (age 4-5 weeks) and BMDCs collection (age 6 weeks). All animal work was approved by the University of Queensland Institutional Animal Care and Use Committee and performed under the certificate guideline.

Antibodies Anti-mouse CD11c-APC, anti-mouse CD80-FITC/CD86-PE/MHC II-FITC/CD40-FITC/ CD4-FITC, anti-mouse CD8a and Cy3-goat anti-rat IgG were purchased from Biolegend; SIINFEKL-H2kb-PE and Mouse IgG isotype control (FITC) were purchased from Invitrogen; Mouse IgG isotype control (PE) was purchased from Abcam.

Cells RAW 264.7 and HEK 293T cells were cultured in DMEM medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. E.G7 cells were cultured in G418 selective medium (RPMI 1640 media with 10% FBS, 400 μg/mL G418, 0.0035% 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin) for 2 weeks. The RPMI 1640 media with 10% FBS, 0.0035% 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin was used for maintenance. For the culture of primary BMDCs, the mice were euthanised and the bone marrow cells (BMCs) in the femur and shin were flushed and collected in PBS. The

tissue residue in BMCs was cleaned by cell strainer and the red blood cells (RBCs) were removed by RBC lysis buffer. The acquired and cleaned BMCs were then cultured in RPMI 1640 media with 10% FBS, 20 ng/mL GM-CSF, 10 ng/mL IL-4, 100 U/mL penicillin, and 100 μg/mL streptomycin. Every 2-day, half of the cell culture media was removed and replenished with fresh media described above. On day 6 after cells seeding, the non-adherent BMCs (mainly BMDCs) were collected for further experiments.[1] The acquired BMDCs were further confirmed by staining with CD11c (APC conjugated) antibodies, and the gating strategy for the analysis of BMDCs cells by fluorescence-activated cell sorting (FACS) was shown in Figure S5B.

The plasmid DNA and primers The plasmid DNA encoding enhanced green fluorescent protein (pEGFP) was purchased from CLONTECH and amplified in *E. coli* and purified by NucleoBond Xtra Maxi Kit (Macherey-Nagel, endotoxin-free).[26] The DNA of ovalbumin (OVA)[48] was cloned into pcDNA 3.1 vector (Table 1) in Tianjin Medical University Cancer Institute & Hospital, China, and amplified in *E. coli* and purified by NucleoBond Xtra Maxi Kit (Macherey-Nagel, endotoxin-free). In addition, the relative OVA mRNA level was determined by RT-PCR test with primer sets of mOVA-RT and mouse GAPDH (Table 1) as housekeeping gene from Integrated DNA Technologies (IDT).[49]

Table 1. The primers sequence for the construction of pcDNA 3.1/OVA plasmid (pOVA) and the RT-PCR test of OVA mRNA (mOVA) and mouse GAPDH.

Primer name	Sequence
pOVA-UP-EcoR1	GAAGAATTCGCCACCATGGGCTCCATCGGTGCAGC
pOVA-DN-Xho1	GCCCTCGAGTCAAGGGGAAACACATCTGC
mOVA-RT-F	AGAAATGTCCTTCAGCCAAGCTC
mOVA-RT-R	GCCCATAGCCATTAAGACAGATGTG
GAPDH-F	TCTGGAAAGCTGTGGCGTG
GAPDH-R	CCAGTGAGCTTCCCGTTCAG

The cellular uptake of ACP NPs

The cellular uptake of free dsDNA-Cy5, CP-Cy5, and ACP-Cy5 was examined and quantified using FACS (CytoFLEX, Beckman Coulter). In brief, RAW 264.7 cells or bone marrow DCs (BMDCs) were seeded in 24-well plates at a density of 5×10^4 per well and cultured for 24 h at 37 ℃ in a 5% CO_2 humid incubator. Then 10 μg/mL ACP-Cy5 (with 50 nM DNA-Cy5) was added and co-cultured for 1 and 4 h. After culture, the medium was discarded, and the cells were collected and washed three times with PBS and re-suspended in 4% PFA solution for FACS analysis. Meanwhile, the free DNA-Cy5 and CP-Cy5 NPs were used as control groups following the same protocol.

The activation of BMDCs by ACP NPs

The BMDCs were seeded in 24-well plates at a density of 1×10^5 per well and cultured for 24 h at 37 ℃ in a 5% CO_2 humid incubator. Then 10 or 50 μg/mL of CP, ACP, or 1 μg/mL of LPS was added and the cells and co-cultured for 24 h. The cells were collected and washed three times with PBS and labelled by CD11c-APC antibody (Biolegend), CD80-FITC, CD86-PE, MHC II-FITC, and CD40-FITC (Biolegend) antibodies in PBS with 1% BSA at 4℃ for 1 h following manufacturer protocol. The cells were again washed three times with PBS and resuspended in 4% PFA solution for FACS analysis. The mouse IgG isotype control (FITC, Invitrogen or PE, Abcam) was used as the control for FACS analysis.

In vitro delivery of pEGFP to RAW 264.7 cells by ACP NPs

The transfection efficiency of ACP-pEGFP NPs and EGFP expression were investigated. In brief, RAW 264.7 cells were seeded in 24-well plates at a density of 5×10^4 per well and cultured for 24 h at 37 ℃ in a 5% CO_2 humid incubator. CP-pEGFP or ACP-pEGFP was added to reach the final 1.0 μg/mL of pEGFP and co-cultured for 6 h. The cells were washed with 37 ℃ pre-warmed PBS and fresh media was added for incubation at 37 ℃ for 48h. Then cells were collected and washed three times with PBS and resuspended in 4% PFA solution for FACS analysis (channel FITC). Meanwhile, the transfection dose of ACP-pEGFP was optimised, the ACP NPs with different weight % of pEGFP in the range of 2.5 to 50 % were transfected and tested after 48 h treatment. Here lipofectamine 2000 (L2K) was used as a comparison following the similar protocol provided by the manufacturer to transfect the equivalent dose of pEGFP.

In vitro delivery of pOVA model vaccine by ACP NPs

The antigen presentation through MHC-I after pOVA vaccines treatment was further tested on BMDCs. In brief, BMDCs were seeded in 24-well plates at a density of 1×10^5 per well and cultured at 37 ℃ in a 5% CO_2 humid incubator overnight. Then 10 μg/mL of CP-pOVA or ACP-pOVA with 1 μg/mL of pOVA was added and the cells co-cultured for 48 h. The cells were collected and washed three times with PBS and stained by CD11c-APC (Biolegend) and SIINFEKL-H2kb-PE (Invitrogen) antibodies in PBS with 1% BSA at 4 ℃ for 1 h. The stained cells were then resuspended in 4% PFA solution for FACS analysis.

The cytotoxicity of ACP NPs

RAW 264.7 or HEK 293T cells were seeded in 96-well plates at 1×10^4 cells per well in 100 μL media overnight. Triplicate wells of cells were incubated with CP or ACP NPs at the concentration of 10, 25, 50, 100 and 150 μg/ml at 37 ℃ in 5% CO_2 for 24 h. The viability of cells was determined by MTT assay and the absorbance of each well was measured at 570/630 nm using the plate reader (INFINITE M PLEX, Tecan).

Measurement of anti-OVA IgG, IgG1, and IgG2a antibody by ELISA

The anti-OVA antibody in serum was determined by indirect ELISA as previously described with modifications.[5] Briefly, the 96-well plates were coated with 100 μl of OVA (10 μg/ml) in the carbonate-bicarbonate buffer (0.1 M, pH 9.6) per well overnight at 4°C. The plates were blocked with 5% non-fat milk powder at room temperature for 2 h. The serum samples were diluted in PBS and added in plate and then incubated with at room temperature for 1.5 h. Afterwards, the plates were incubated with 100 μl of 1:5000 diluted horseradish peroxidase-conjugated anti-mouse IgG, IgG1, or IgG2a at room temperature for 1 h. At last, the plates were incubated at room temperature for 15 min with 100 μl of TMB Chromogen Solution (Thermo Scientific). The reaction was stopped by 100 μl of 1 M H_2SO_4, and the absorbance at 450 nm was measured by a plate reader (INFINITE M PLEX, Tecan).

Measurement of IFN-γ production by SIINFEKL recalled splenocytes

The IFN-gamma (IFN- γ) cytokine secreted during the antigen-recall of splenocytes were analysed through ELISA tests as described previously.[6] On day 7 after the last immunisation, the mice were euthanised (n = 3 animals per group) and their spleen lymphocytes were isolated using RPMI 1640 through cell strainers (70 μm, FALCON) and treated with RBC lysis buffer (BioLegend). Splenocytes were then counted and plated at 1×10^5 cells per well in 48-well plate followed by antigen recall with 2.5 μg of OVA H-2Kb-restricted CTL epitope (OVA257–264, SIINFEKL, Sigma-Aldrich) for 48 h. The IFN-γ concentration in the cell culture supernatant was measured by mouse IFN-gamma ELISA kit (Invitrogen) follow the manufacture protocol.

Results

The size and PdI of CP NPs with different amounts of ATP or DNA

Table S2. The average size and PdI of CP NPs with different amounts of ATP or DNA

CP NPs with	0% ATP	12.5 % ATP	25 % ATP	50% ATP	75% ATP	50% ATP-DNA
Size (nm)	697.2	405.4	231.6	101.2	109.9	130.6
PdI	0.243	0.349	0.468	0.319	0.375	0.385

The loading efficiency of plasmid DNA and ATP on ACP NPs

Table S3. The loading efficiency of ATP and pEGFP on CP NPs

	pEGFP amount (μg/mL)			
	0	50	100	200
ATP loading efficiency	93.8%	93.7%	93.8%	93.9%
pEGFP loading efficiency	-	100%	96.6%	93.7%

The amount of CP and ATP were 1 mg/mL and 500 ug/mL, respectively.

The particle size change of CP-pEGFP and ACP-pEGFP NPs in the medium during 72h period

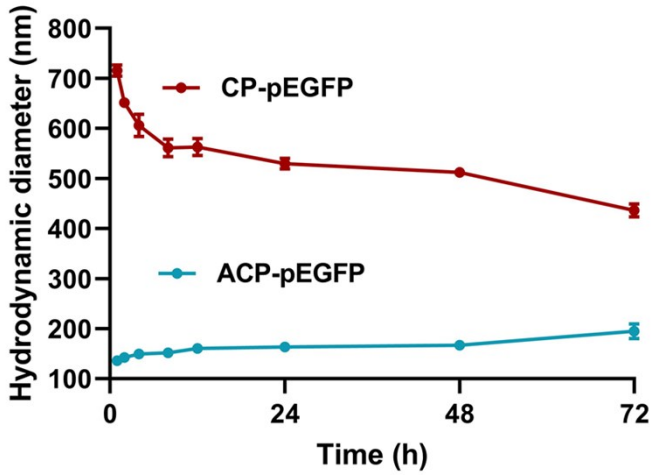

Figure S1. The hydrodynamic diameter of CP-pEGFP and ACP-pEGFP NPs in cell culture medium with 10% FBS for 72h period.

The ATP release from ACP NPs in PBS buffer

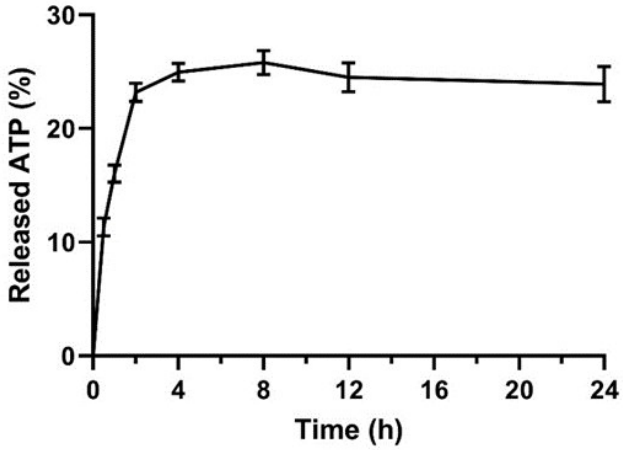

Figure S2. The ATP release profile of ACP NPs in PBS buffer (pH = 7.4) in 24 h.

The cellular uptake of CP and ACP NPs by RAW 264.7 cells

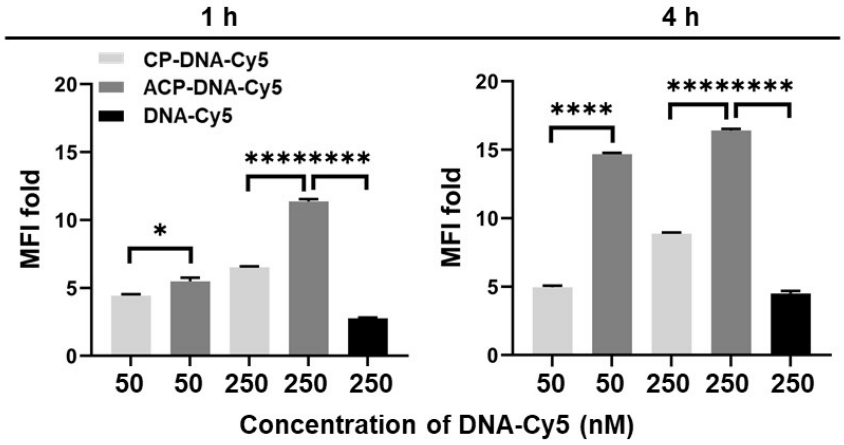

Figure S3. The FACS analysis for cellular uptake efficiency of CP-Cy5-DNA and ACP-Cy5-DNA by RAW 264.7 cells after 1 and 4 h incubation. The MFI fold was calculated by normalising the MFI of the untreated cells as 1.

The dissolve of CP-DNA-Cy5 and ACP-DNA-Cy5 NPs in physiological (7.4), endosomal (6.5), and lysosomal (5.7) buffers and the release profile of DNA-Cy5

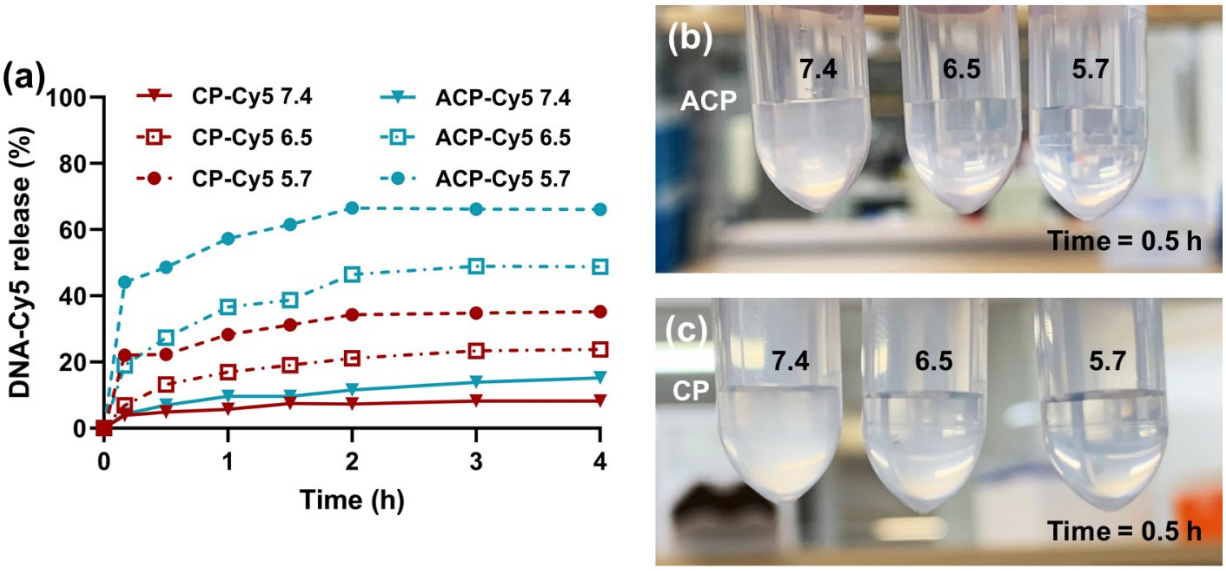

Figure S4. The CP-DNA-Cy5 and ACP-DNA-Cy5 NPs dissolve and DNA-Cy5 release in phosphate buffers (pH = 7.4, 6.5, and 5.7). (a) The DNA-Cy5 release profile of CP-DNA-Cy5 and ACP-DNA-Cy5 NPs in pH 7.4, 6.5, and 5.7 buffers in 4 h. The images of (b) ACP-DNA-Cy5 and (c) CP-DNA-Cy5 NPs incubated in phosphate buffers (pH = 7.4, 6.5, and 5.7) for 30 min.

The activation of mouse BMDCs by treatment with ATP, CP NPs, and ACP NPs

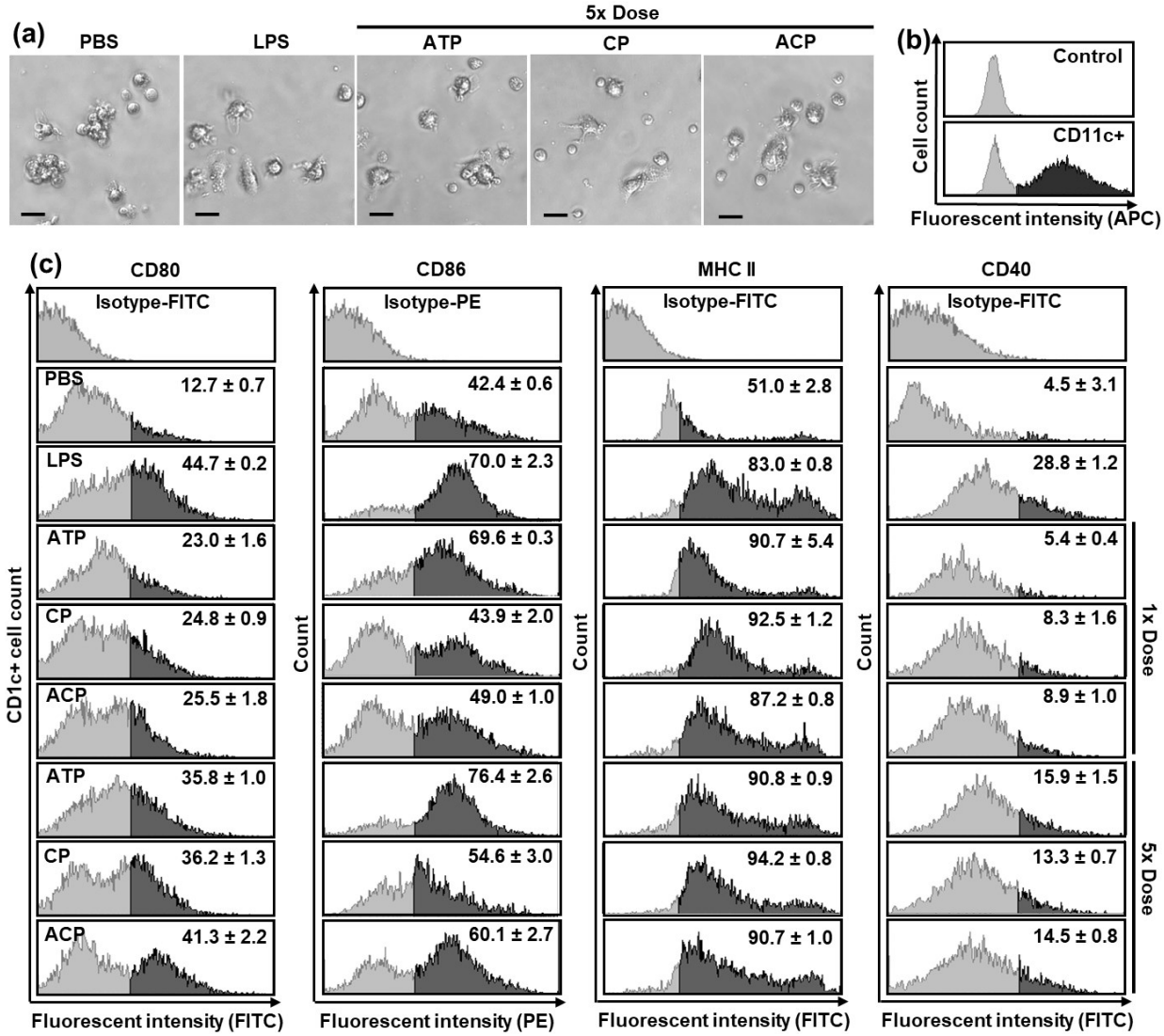

Figure S5. The activation of BMDCs by ATP, CP NPs, and ACP NPs. (a) The morphology of BMDCs after 24 h treatment of PBS, 1 μg/mL LPS, 5x dose of ATP, CP NPs, or ACP NPs. Scale bar = 20 μm. (b) The gating strategy of CD11c+ (APC fluorescent) BMDCs. (c) The FACS histogram and the positive cell percentage of the CD80, CD86, MHC II, and CD40 antibodies treated CD11c+ BMDCs after 24 h incubation with PBS, 1 μg/mL LPS, ATP, CP NPs, or ACP NPs. The 1x dose of ATP was 5 μg/mL and CP was 10 μg/mL, while ACP was the combination of both.

The nodes of s.c. injected pOVA, CP-pOVA NPs, and ACP-pOVA NPs.

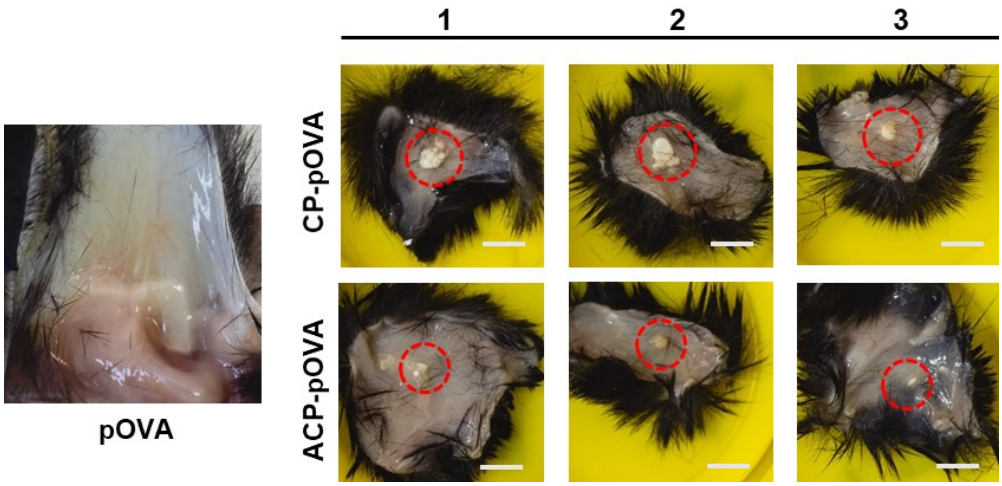

Figure S6. The CP and ACP NPs residue at the injection site 24 h after s.c. injection (n = 3).

The weight of mice tumours

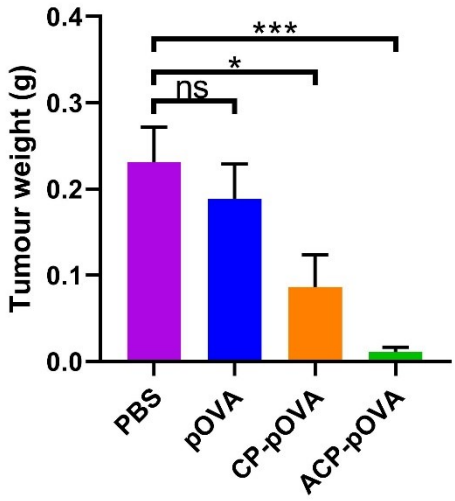

Figure S7. The tumour weight of mice in different groups at day 46 post prime.

Reference

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