Electronic Supplementary Information (ESI)

Bio-engineered Cell Membrane Nanovesicles as Precision Theranostics for Perihilar Cholangiocarcinoma

Xiaojie Zhang,[‡a] Yang Zhang,[‡b] Yunming Zhang,[‡b] Peng Lv,[b] Pengfei Zhang,[b] Chengchao Chu,[b] Jingsong Mao,[a] Xiaoyong Wang,[*b] Wengang Li,[*a] Gang Liu[*b]

[a] Department of Hepatobiliary surgery, Xiang'an hospital of Xiamen university, school of Medicine, Xiamen university, Xiamen 3610102, Fujian, China. E-mail：lwg11861@163.com (Wengang Li)

[b] State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen, 361102, China.
E-mail: gangliu.cmitm@xmu.edu.cn (Gang Liu); wangxy@xmu.edu.cn (Xiaoyong Wang)

[‡]These authors contributed equally to this work.

1. Materials and method

1.1. Materials

Indocyanine green (ICG) was purchased from J&K Scientific (Beijing, China). 4-nitrophenyl chloroformate 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), thiophene-2-thiol, 4, 6-Dia-midino-2- phenylindole (DAPI), were purchased from Sigma Aldrich (MO, USA). RPMI 1640 (Roswell Park Memorial Institute 1640), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from HyClone (Xiamen, China). Golgi body staining, Lipo6000TM was purchased from Beyotime (Xiamen, China). The Affibody® molecule $_{ZHER2:342}$ gene fusing with signal peptide and transmembrane sequence were subcloned into a pcDNA 3.1 vector in Shanghai Science Biological Technology, Shanghai, China (Fig. S1).

The human breast cancer cell line (BT474), Human normal hepatocyte (LO2), and the human biliary tract cancer cell line (SK-ChA-1), Human embryonic kidney 293T cell (HEK 293T) were purchased from American Type Culture Collection (ATCC). BALB/c nude male mice (6-8 weeks old, average body weight 20 g) were obtained and raised at Animal Care and Use Committee (CC/ACUCC) of Xiamen University (Xiamen, China).

1.2. Plasmid construction, cell culture, and transfection

Terminal signal peptide DNA sequence and transmembrane peptide DNA sequence were linked together on the two ends of affibody® molecule $Z_{HER2:342}$ gene sequence. The transmembrane peptide DNA sequence was linked with GFP gene sequence at the carboxyl terminus. The whole DNA sequence was synthesized and then inserted into two Hind III / BamH I enzyme sites of pcDNA 3.1 vector (Table S1 and Fig. S1). In this study, all kinds of cells were cultured within a humidified (5% CO_2 and 95% air) atmosphere at 37 ℃. For fluorescent detection, the HEK 293T cells were transfected with recombinant plasmid using Lipo 6000^{TM} in accordance with the manufacturer's instructions. After incubated for 48 h, the transfected cells were incubated with DAPI and Golgi body staining for

10 min, sequentially. Then, the cells were washed with Phosphate buffer saline (PBS) and observed by confocal laser scanning microscopy (CLSM, Olympus, Japan).

1.3. Preparation of ICG loaded A-NVs

Biomimetic NVs were prepared based on the previous laboratory's study.[1] The harvested cells were removed cellular debris and DMEM medium by PBS mixed with a final concentration of 1 mM protease inhibitor. Then, the cells were collected in a sterile 1.5 mL EP tube and resuspended in PBS with protease inhibitor. The cells were sonicated by low-power (20 W, 30 s). The nanovesicles were separated by multi-steps density gradient ultracentrifugation, the whole cells (2,000 rpm, 10 min) and cell debris (5,000 rpm, 15 min) were discarded in the sterile 1.5 mL EP tube, respectively. Then the supernatant was regathered and ultra-centrifuged (15000 rpm, 60 min) in the sterile 1.5 mL EP tube. Finally, the product was resuspended with 200 μL PBS. To further purify uniform nanovesicles, the product was sonicated (20 W, 10 s) and introduced to a Mini-Extruder (Avanti Polar Lipids) equilibrated in PBS (200 nm pore-sized membrane filters). All operations are maintained in the ice bath. To validate the expression of affibody® molecule $Z_{HER2:342}$, we purified proteins from harvested cells membrane and control for western blotting (Fig. S2).

For ICG-loaded nanovesicle preparation, the farinose ICG was dissolved in ultrapure water to make an accurately concentration of 1 mg/mL. And then it's diluted to make a series of solutions (range from 0 to 250 μg/mL) for making function at 780 nm UV-vis-NIR absorbance. ICG was added into A-NVs or NVs during ultrasonic vibration (20 W, 30 s) and standing 20 min generate nanoscaled A-NVs@ICG or NVs@ICG. After centrifuging (15,000 rpm, 60 min), we measured the concentration of free ICG using regression equation and calculated the ICG loading efficiency (Fig. S3).

1.4. Characterization of A-NVs@ICG

The morphology of the A-NVs was examined by a Transmission Electron Microscopy (TEM, Philips CM200 FEG). The UV-vis-NIR absorbance was measured by a UV-vis-NIR spectrometer (THERMO). The zeta potential and hydrodynamic size of NPs were measured by dynamic light scattering (DLS, Zeta Sizer/NanoZS90). The FL imaging was measured by a Xenogen IVIS Kinetic (Caliper Life Sciences) (ICG: 0, 1.25, 2.5, 5.0, 10.0, 20.0 μg/mL). The PA imaging was measured with commercial Visualsonics LAZR-X Vevo (Fujifilm, Japan) using an excitation wavelength of 780 nm (ICG: 0, 6.2, 12.25, 25, 50, 100 μg/mL). The photothermal effect of A-NVs@ICG at different concentrations (ICG: 0, 2.5, 5, 10, 20, 40, 80 μg/mL) were irradiated with 800 nm laser (1.0 W/cm^2, Sintec Optronics Technology, Singapore) for 5 min. Simultaneously, the temperature variation of the solution (each 100 μL) and thermal images were recorded at an interval of 5 s using a FLIR Ax5 camera (FLIR Systems Inc, Wilsonville, OR). A-NVs@ICG were suspended in PBS and measured in triplicate.

1.5. HER2 expression quantity detection of PHCC

To further determine the treatment for PHCC, we used undefined cells (SK-ChA-1), HER2-positive (BT474)[1], and HER2-normal (LO2) [2] for western blotting. In this study, all kinds of cells were cultured within a humidified (5% CO_2 and 95% air) atmosphere at 37 ℃. And then, the total protein was collected and measured by BCA kit and SDS-PAGE. Subsequently, the samples were transferred onto polyvinylidene fluoride (PVDF) membrane for probing with an anti-HER 2 (1 : 1000, Beytime, Cambridge, UK) antibody and β-actin antibody (1 : 1000, Abcam, Cambridge, UK).

1.6. *In vitro* PHCC target ability verification of A-NVs

To determine A-NVs targeting to tumor cells, SK-ChA-1 and MCF-7 cells were seeded in confocal dishes at a density of 2×10^5 cells per well for 12 h. Next, the SK-ChA-1 and MCF-7 cells were incubated with RPMI 1640 (without 10% FBS) containing A-NVs (5 μg/mL proteins) for 6 h, respectively. The cells were rinsed 3 times with PBS buffer and fixed with 4% paraformaldehyde for 15 min at room temperature. After being stained with the

DAPI for nuclear staining and then removed unbound nanoparticles with PBS. Finally, the cellular binding was observed with CLSM (Objective lens: 63X).

1.7. *In vitro* ICG delivery detection

SK-ChA-1 cells were seeded in confocal dishes at a density of 2×10^5 cells per well. RPMI 1640 (without 10% FBS) containing 20 μg ICG (free ICG, NVs@ICG, or A-NVs@ICG) was used for evaluating the cellular uptake ICG assay of SK-ChA-1 cells.[3] Additionally, free affibody was used for blocking the binding site of HER2 on SK-ChA-1 cells membrane. Finally, the *in vitro* cellular uptake assay was observed with CLSM (Objective lens: 63X).

1.8. *In vitro* cellular toxicity test and antitumor activity

The *in vitro* cytotoxicity of A-NVs@ICG against SK-ChA-1 cells was tested MTT assay with or without 808 nm laser irradiation. Firstly, SK-ChA-1 (2.0×10^4 cells per well) were seeded in 96-well culture plates with RPMI 1640 medium containing 10% FBS for 12 h. Then, the cells were treated with PBS, A-NVs, free ICG, NVs@ICG, or A-NVs@ICG (equivalent ICG concentration: from 5 to 40 μg/mL) for 6 h. Subsequently, the wells were washed 3 times with PBS to remove unbound nanoparticles. Finally, the cells were re-cultured with fresh medium for 12 h and further detected by MTT assay. Additionally, the SK-ChA-1 cells were cultured as above and further irradiated by laser (1 W/cm^2, 808 nm) for 5 min. After 12 h of incubation, the cell viability was also measured using the MTT assay.

1.9. Animals and tumor model

All animal studies were performed under the animal use and care regulations approved by Center of Animal Care and Use Committee, Xiamen University. For the *in vivo* multimodal imaging, SK-ChA-1 cells (1×10^7 cells/site) suspended in PBS were subcutaneously injected into the flank region of female BALB/c athymic nude mice (n=4/group, about 20 g). The volume of the tumors reached 100 mm^3 was performed for fluorescence imaging and photoacoustic imaging (about 20 days after implant). All of the tumor volume was calculated according to the following formula: tumor volume= (length) $\times$ (width)^2 /2.

1.10. Multimodal imaging *in vivo* and biodistribution

The *in vivo* fluorescence imaging was respectively monitored using Xenogen IVIS Kinetic before and after intravenously administered with the equivalent ICG dose of A-NVs@ICG NVs@ICG and free ICG at pre, 1, 6, and 9 h post-injection. Besides, the mice were sacrificed and harvested tumor tissues and major organs (heart, liver, spleen, lung, and kidney) from the body at 9 h post-injection for investigating the biodistribution of the ICG *in vivo*. Instantly, the isolated organs and tumor tissues were acquired fluorescence imaging and quantified to reveal the distribution of ICG on A-NVs@ICG, NVs@ICG, and free ICG groups.

To study the photoacoustic performance of A-NVs@ICG in tumor environment responsiveness, each SK-ChA-1 tumor-bearing mice (4 mg/kg ICG) was also intravenously administered with A-NVs@ICG, NVs@ICG, and free ICG (Each containing 80 μg of ICG per mouse). Before the photoacoustic experiment, the mice were anesthetized and gathered the signal on tumor area at the excitation wavelength of 780 nm. After intravenous injection of A-NVs@ICG, NVs@ICG, and free ICG (Each containing 80 μg of ICG per mouse), photoacoustic imaging was obtained at pre, 1, 6, and 9 h.

1.11. Multimodal imaging-guided *in vivo* phototherapy

A-NVs@ICG, NVs@ICG, and free ICG were intravenously administered into SK-ChA-1 tumor-bearing mice (n=4 /group). BALB/c nude mice with SK-ChA-1 tumors were irradiated with 808 nm laser (1.0 W/cm^2) for 5 min. Meanwhile, the real-time temperature and thermal images were obtained from a FLIR Ax5 camera.

To monitor the change of tumor volume and body weight, we recorded the size of tumor and body weight every other day. We took photos of isolated tumors from the treated mice at the end of trial. Finally, treated mice bearing SK-ChA-1 tumors whose volume exceeded 1000 mm^3 were euthanatized according to the animal experiment instructions.

1.12. Histological analysis

Treated BALB/c nude mice bearing SK-ChA-1 tumors were sacrificed at 12 days post-treatment. Synchronously, the tumors and major organs (including heart, liver, spleen, lung, and kidney) were harvested and fixed in 4% paraformaldehyde solution. The tissues were paraffin embedded, solidified, and sectioned into a thickness of 5 μm for Haematoxylin and Eosin (H&E) staining. The results were observed using an Intelligent biological microscope Olympus BX53 (Olympus).

1.13. Hemolysis analysis of A-NVs@ICG

Hemolysis assay was executed using red blood cells (RBCs). We collected the peripheral whole blood collected from the ocular vein of BALB/c nude mice, simultaneously. It was flowed into the inwall of equally distributed heparin sodium of the tube. The erythrocytes were washed 5 times using saline and collected at 1500 rpm for 15 min. Then, 100 μL of erythrocytes (4% v/v) were added to 1 mL of A-NVs@ICG dispersions with different concentrations of ICG (varied from 5 to 40 μg/mL) for each sample in triplicates. Meanwhile, deionized (DI) water and saline were respectively used as positive and negative control. After incubated for 3 h at 37 °C, the supernatant was separated at 12,000 rpm for 15 min and measured by UV-vis-NIR spectrometer at 540 nm absorbance. The hemolysis percentage was calculated using the following formula[4]:

Hemolysis (%) = $(A_0-A_N)/(Ap-A_N)$ x 100%

A_0, A_N, and A_P, severally represent the absorbance of A-NVs@ICG group to the erythrocyte suspension, the absorbance of saline group and DI water group.

1.14. Serum biochemical analysis

Based on the standard collection procedures, the peripheral whole blood was collected from the ocular vein of BALB/c nude mice before and after intravenously administered with A-NVs@ICG at 48 h post-injection. After standing for 1 h at room temperature, the serum samples were separated twice from whole blood (about 0.5 mL each mouse) at 3,000 rpm for 10 min and then measured for biochemistry tests and hepatotoxicity evaluation. Finally, Hepatic indicators were obtained using a Biochemical automatic analyzer (Type 7170, Hitachi, Japan).

1.15. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA, USA). All results are expressed as mean ± SD for each group. Two-way analysis of variance (ANOVA) was used to determine the significant differences among multiple groups, and $P < 0.05$ was considered statistically significant.

2. Results and discussion

Table S1. Recombinant plasmid sequence

Name	DNA sequence
$Z_{HER2:342}$ Affibody	GTGGACAACAAGTTCAACAAGGAGATGCGCAACGCCTACTGGGAGATCGCCCTGCTGCCCAA CCTGAACAACCAGCAGAAGCGCGCCTTCATCCGCAGCCTGTACGACGACCCCAGCCAGAGCG CCAACCTGCTGGCCGAGGCCAAGAAGCTGAACGACGCCCAGGCCCCCAAG
Signal peptide	ATGAATTTACAACCAATTTTCTGGATTGGACTGATCAGTTCAGTTTGCTGTGTGTTTGCT
Transmembrane peptide	TTATGGGTCATCCTGCTGAGTGCTTTTGCCGGATTGTTGCTGTTAATGCTGCTCATTTTAGCAC TGTGG
Linker	GGTGGTGGAGGATCAGGTGGTGGTGGTTCTGGTGGAGGTGGAAGT

PcDNA 3.1 Vector

Fig. S1 Schematic illustration of the Signal peptide (Sp)-Affibody-Transmembrane peptide (Tp)-GFP construct, which is expressed onto the cell membrane.

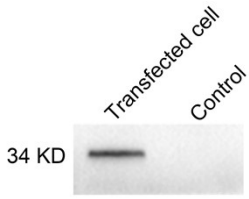

Fig. S2 Western blotting analysis of affibody expressed on HEK 293T cytomembrane after transfected recombinant pcDNA 3.1 vector.

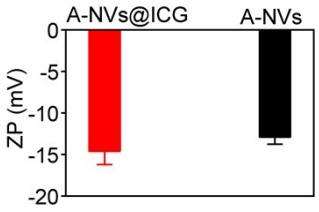

Fig. S3 Zeta potential (ZP) analysis of A-NVs@ICG and A-NVs showing successful ICG encapsulating in A-NVs.

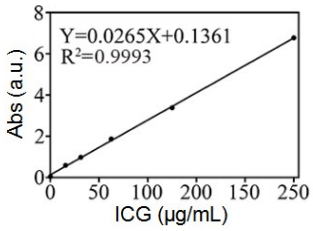

Fig. S4 Fitting curve of the UV-vis-NIR absorbance of free ICG at 780 nm as the function of concentration in the range from 0 to 250 μg/mL.

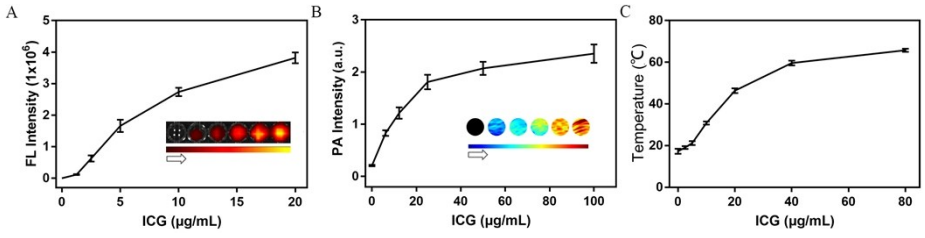

Fig. S5 (A) FL signal intensity and images and (B) PA signal intensity and images of free ICG with increasing ICG concentrations. (C) The temperature variation of A-NVs@ICG with gradient concentrations was monitored under 808 nm laser irradiation (1.0 W/cm^2).

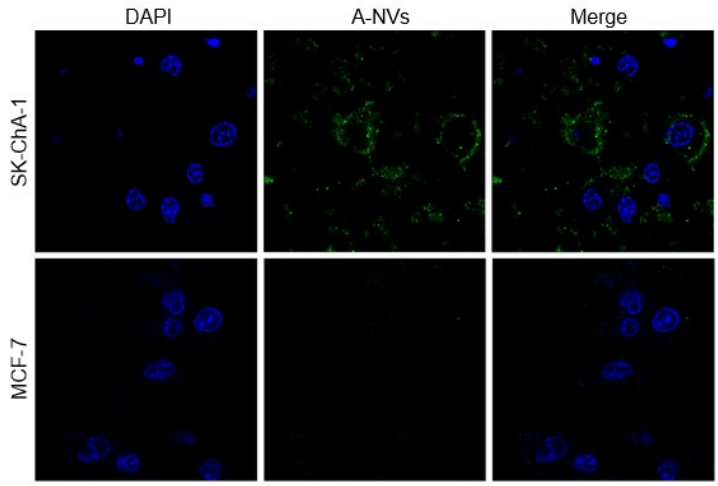

Fig. S6 Cellular binding assay of A-NVs to SK-ChA-1 cells (HER2-overexpressing) and MCF-7 cells (HER2-negative) (Objective lens: 63X).

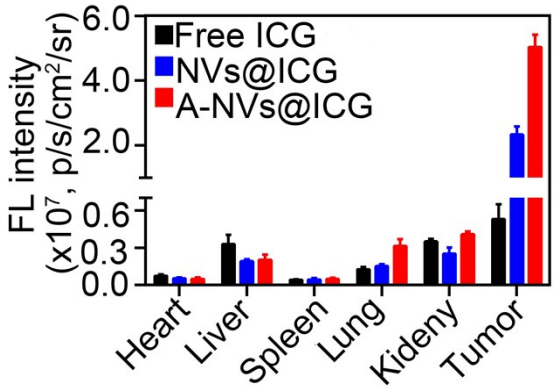

Fig. S7 Semiquantitative biodistribution of free ICG, NVs@ICG, and A-NVs@ICG in SK-ChA-1 bearing nude mice measured by the averaged fluorescence intensity of each organ at 9 h post-injection.

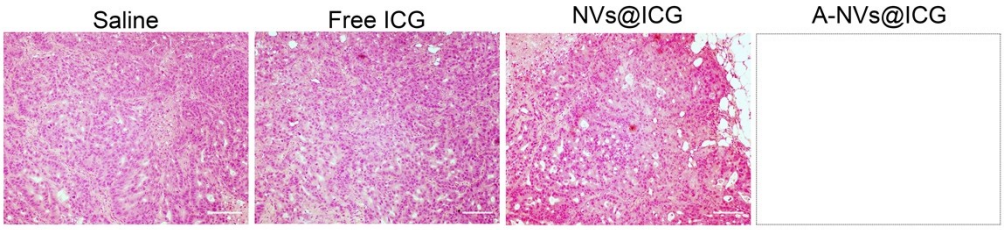

Fig. S8 H&E staining of tumors harvested from the mice at 12 days post-irradiation. Scale bar: 100 μm.

References

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