Self-Assembled Peptido-Nanomicelles as An Engineered Formulation for Synergy-Enhanced Combinational SDT, PDT and Chemotherapy to Nasopharyngeal Carcinoma

Electronic Supplementary Information

1. Materials

The amphiphilic peptide ($C_{18}GR_7RGDS$) was obtained from DG Peptides Co., Ltd. (Hangzhou, China), and Rose Bengal (RB) was purchased from Aladdin Industrial Corporation (Shanghai, China). 2',7'-Dichlorofluorescein diacetate (DCF-DA) was from MACKLIN (Shanghai, China). Cell Counting Kit (CCK-8), H&E Staining Kit, paraformaldehyde, DNase I, phosphate buffer solution (PBS), penicillin and streptomycin were obtained from Beyotime Biotechnology (China). Roswell Park Memorial Institute-1640 (RPMI-1640), fetal bovine serum (FBS) and trypsin were purchased from GIBCO. In situ cell death assay fluorescein was purchased from Roche (Germany), and LIVE/DEAD Viability Kit was obtained from Life Technologies (USA). ELISA Intact Kit Mouse IL-6, TNF-α and IFN-γ was purchased from Komabiotech (Seoul, Korea). Milli-Q water was used as ultrapure water (Millipore, USA). All chemicals were of analytical grade and no further purification was required.

2. Methods

2.1 Fabrication and characterization of RBNs

RBNs were fabricated via a sonication-promoted self-assembly protocol of the amphiphilic peptide.[S1] Briefly, RB and the amphiphilic peptide were respectively dissolved in the ultrapure water as stock solutions. Then, 1.0 mL of RB (2.0 mg/mL)

and 2.0 mL of peptide (10.0 mg/mL) were mixed together under the mild sonication for 30 min. The resulted mixture was transferred for overnight dialysis (MWCO = 1,000 Dalton). Afterwards, the mixture was diluted for the particle size and zeta measurements on a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instrument Inc., Worcestershire, UK). The RB encapsulation efficiency were determined by measuring the absorbance of RBNs samples with varied RB contents on a UV-Vis spectrometer (Lambda 25, PerkinElmer, USA). With a calibration curve of free RB absorbance at diluted concentrations (2, 4, 6, 8, 10 μg/mL) at 549 nm, the RB concentration was read out, and the RB encapsulation efficacy was accordingly calculated.[S2]

2.2 Cell culture and cytotoxicity

Nasopharyngeal carcinoma cells (CNE-2Z) were cultured in the RPMI 1640 culture medium containing 10% fetal bovine serum and 1% streptomycin-penicillin at 37°C under the atmosphere of 5% CO_2. The CNE-2Z cells were seeded for 48 h incubation in a 96-well plate (1×10^4 cells per well). After the respective co-incubations of CNE-2Z cells with free RB and RBNs at a RB concentration of 0, 10, 20, 40, 60, 80, 100 μg/mL, fresh RPMI 1640 medium (100 μL) together with CCK-8 (10 μL) was added followed by fluorescence analysis on a microplate reader (Varioskan LUX, Thermo, USA) at a wavelength of 450 nm. Cell viability was calculated according to the formula below:

$$cell\ viability = \frac{(OD - OD_{blk})_{ave}}{(OD_{ori} - OD_{blk})_{ave}} * 100\%$$

2.3 Intracellular ROS detection and LIVE/DEAD staining tests

CNE-2Z cells were seeded into 35 mm cell-culturing dishes at a density of 3×10^5 cells per dish. After a 24 h incubation, fresh PRMI 1640 medium containing 20

μg/mL RBNs and RB were added, respectively. After further incubation for 4 h, each batch of cells were washed with PBS and then left in fresh PBS for 5 min for minimized extracellular hydrolysis. Then all groups were incubated with 2 mL of DCF-DA aqueous solution. After loading for 30 min, the buffer was replaced with fresh PBS. The samples were subsequently treated with US (1.0 W, 3.0 MHz, 50%, 3 min) and laser irradiation (808 nm, 1.5 W/cm^2, 3 min). The intracellular ROS generation could be visualized using a CLSM (Nikon A1, Nikon, USA).

As for the LIVE/DEAD staining, the cells were stained with calcein-AM (1.0 μM) and PI (2.0 μM) respectively for live and dead cell detections.[S3] Staining images could be visualized with a fluorescence microscope (Axio Vert A1, ZEISS, Germany) and the corresponding cell viability could be determined using a common CCK-8 assay protocol.

2.4 Quantitative comparisons for respective contribution of SDT and PDT

The CNE-2Z cells were seeded into a 96-well plate (1 × 10^4 cells per well) in 100 μL medium with 48 h incubation. To compare the contributions of SDT and PDT, 20 μg/mL of RBNs was co-incubated with the cells followed by US exposure (1.0 W/cm^2, 1.0 MHz, 50%, 3 min) and laser irradiation (808 nm, 1.5 W/cm^2, 3 min) respectively. The cell viability was accordingly analyzed by a CCK-8 assay. Furthermore, the influence of time duration (1-5 min) for US exposure or laser irradiation were also evaluated, and the cell survivals were also calculated with an identical method.

2.5 In-vivo combinational therapy study of nude mice

Male BALB/c CNE-2Z tumor-xenografted nude mice were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animal experimental procedures have been approved by the Administrative Panel of Wenzhou Medical University.

The nude mice were randomly divided into 5 experimental groups (4 mice for each group) which were intravenously or intra-tumorally injected with RBNs (150 μL, C_{RB} = 350 μg/mL) for therapy groups and PBS (150 μL) for the control according to the treatment program. The US exposure (3.0 W/cm^2, 3.0 MHz, 50%, 3 min) and laser irradiation (808 nm, 1.5 W/cm^2, 3 min) were respectively performed. The tumor size and body weight for each mouse were carefully measured and the images were simultaneously recorded.

2.6 Blood examination, cytokines analysis and histological H&E-TUNEL stainings

After the treatments, the mice were anesthetized and blood was collected into an anticoagulant tube for routine examinations. The major organs (heart, liver, spleen, lung and kidney) and tumor tissues of each group were excised into approximately 4 μm slices on a cryostat section machine (HM525 NX, ThermoFisher Scientific, USA) for H&E staining. The tumor tissues were further performed with TUNEL staining by using a one-step TUNEL apoptosis assay kit for cell apoptosis/necrosis assessments on a fluorescence microscope (AxioVert A1, ZEISS, Germany).[S4] In addition, representative pro-inflammatory cytokines of IL-6 and TNF-α were analyzed using an ELISA kit (Komabiotech, Korea).

2.7 Statistical analysis

Differences between the experimental groups were analyzed using a one-way ANOVA analysis. A p value of < 0.05 was considered to be statistically significant, and the significance was marked as *, ** and *** when $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

3. Figures

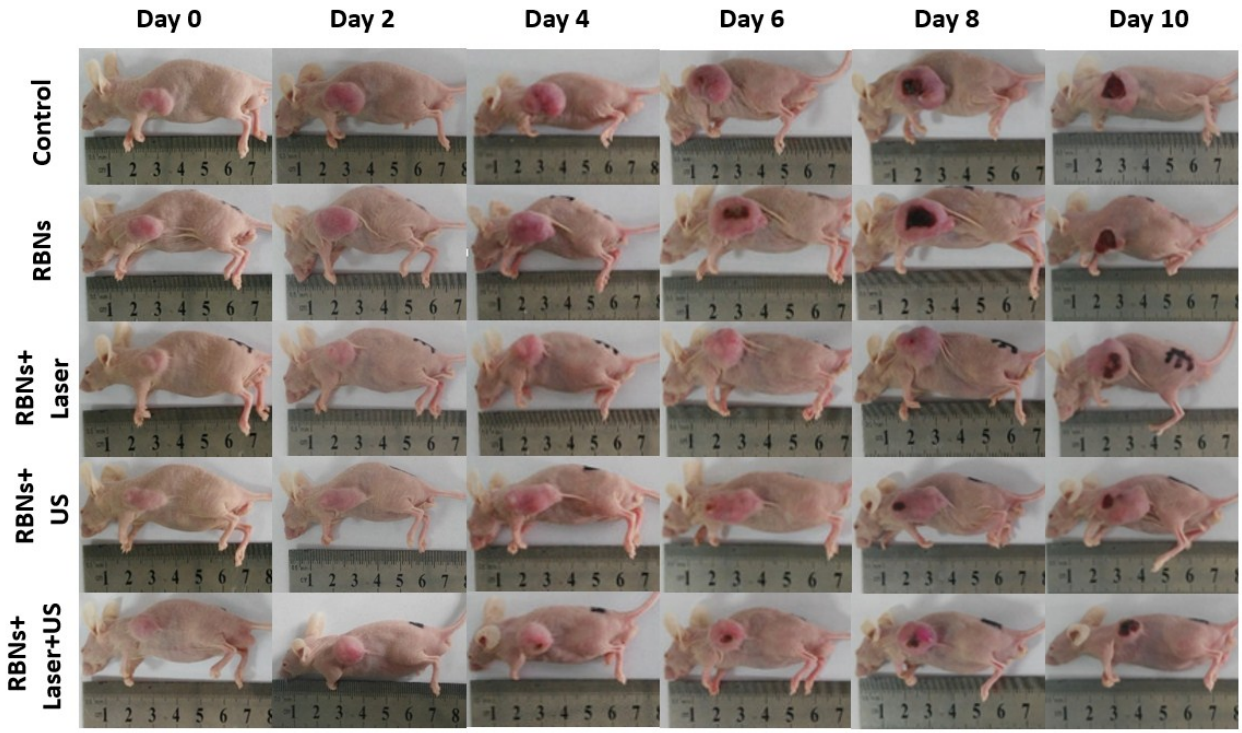

Figure S1. Images of CNE-2Z tumor-xenografted nude mice after the administration of RBNs for synergy-enhanced treatments.

4. References

[S1] Liu, Z.; Li, J.; Jiang, Y.; Wang, D. Multifunctional nanocapsules on a seesaw balancing sonodynamic and photodynamic therapies against superficial malignant tumors by effective immune-enhancement. *Biomaterials* **2019**, DOI: 10.1016/j.biomaterials.2019.119251.

[S2]Zheng, M.; Yue, C.; Ma, Y.; Gong, P.; Zheng, C.; Sheng, Z.; Zhang, P.; Wang, Z.; Cai, L. Single-Step assembly of DOX/ICG loaded lipid–polymer nanoparticles for highly effective chemo-photothermal combination therapy. *ACS Nano* **2013**, *7*, 2056-67.

[S3] Huang, P.; Qian, X.; Chen, Y.; Yu, L.; Lin, H.; Wang, L.; Zhu, Y.; Shi, J. Metalloporphyrin-encapsulated biodegradable nanosystems for highly efficient magnetic resonance imaging-guided sonodynamic cancer therapy. *J. Am. Chem. Soc.* **2017**, *139*, 1275-84.

[S4] Xu, Y.; Liang, X.; Bhattarai, P.; Sun, Y.; Zhou, Y.; Wang, S.; Chen, W.; Ge, H.; Wang, J.; Cui, L.; Dai, Z. Enhancing Therapeutic Efficacy of Combined Cancer Phototherapy by Ultrasound-Mediated In Situ Conversion of Near-Infrared Cyanine/Porphyrin Microbubbles into Nanoparticles. *Adv. Funct. Mater.* **2017**, *27*, 1704096.