



## Chemical Communications

### COMMUNICATION

#### Supporting Information

#### Immuno-potentiating vehicle made of mesoporous silica-zinc oxide micro-rosettes with enhanced doxorubicin loading for combined chemoimmunotherapy

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Table S1 Amounts of reagents during the synthesis of MS-Zn micro-rosettes

	Zn(CH <sub>3</sub> COO) <sub>2</sub> (g)	DMEA (g)	TEOS (g)
MS-Zn-1	0.55	10	4.98
MS-Zn-2	0.55	10	3.32
MS-Zn-3	0.55	10	1.66

Table S2 Si/Zn molar ratio of the MS-Zn-1, MS-Zn-2 and MS-Zn-3 micro-rosettes

	MS-Zn-1	MS-Zn-2	MS-Zn-3
Si/Zn	7.69 ± 0.11	5.06 ± 0.17	2.52 ± 0.01

Table S3 BET specific surface area, pore volume and diameter of the MS-Zn-1, MS-Zn-2 and MS-Zn-3 micro-rosettes

	S <sub>BET</sub> (m <sup>2</sup> /g)	V <sub>p</sub> (cm <sup>3</sup> /g)	D (nm)
MS-Zn-1	27	0.10	3.32
MS-Zn-2	62	0.24	3.27
MS-Zn-3	195	0.70	3.41

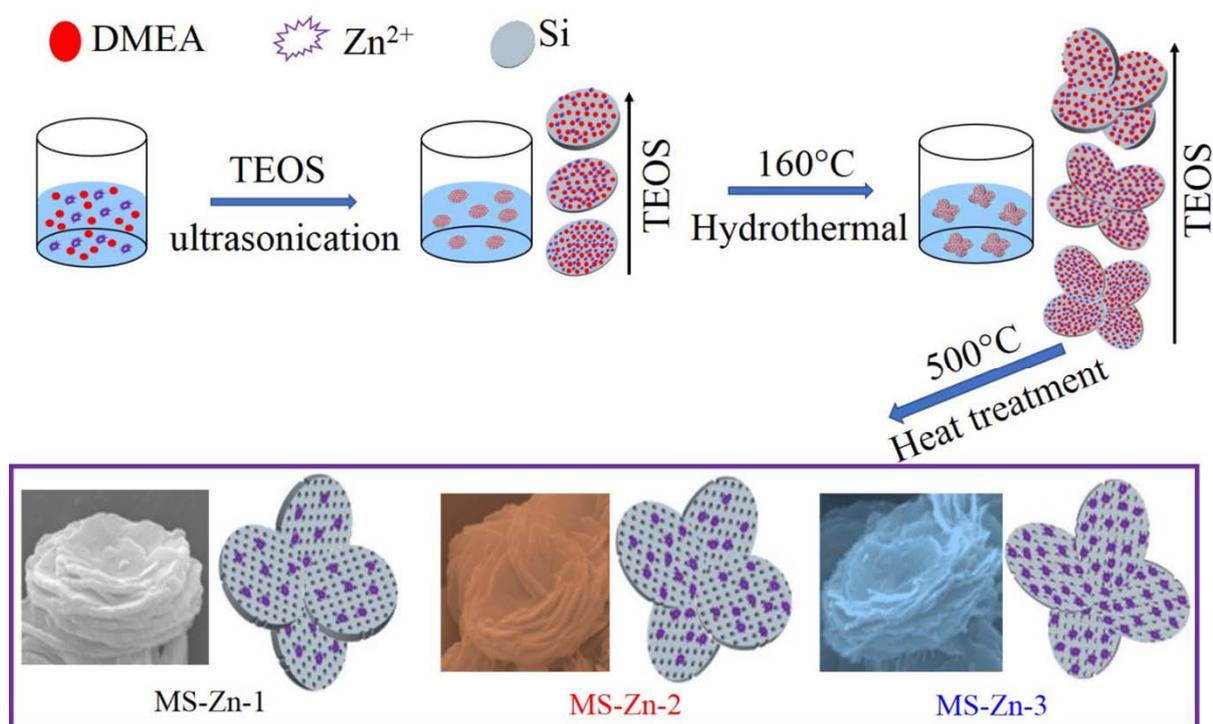


Figure S1 Schematic illustration of the synthesis of MS-Zn micro-rosettes with controllable petal thickness.



Figure S2 SEM images of MS-Zn micro-rosettes loaded with DOX.

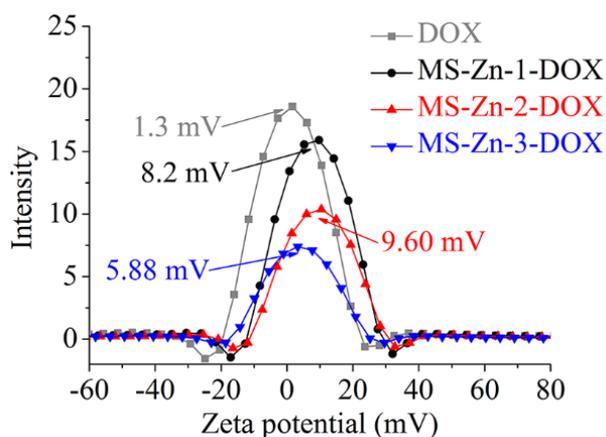


Figure S3 Zeta potentials of free DOX, MS-Zn-1-DOX, MS-Zn-2-DOX and MS-Zn-3-DOX.

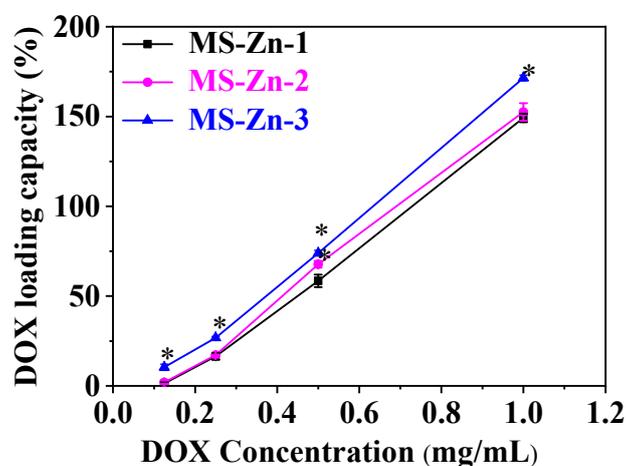


Figure S4 The DOX loading capacity of MS-Zn micro-rosettes with different DOX concentrations ( $n = 3$ ,  $*P < 0.05$ ).

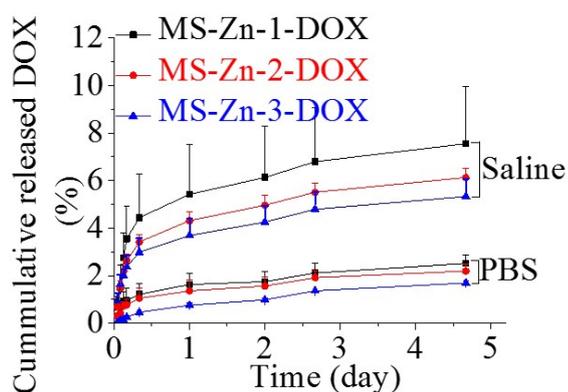


Figure S5 Cumulative release of DOX from MS-Zn-1-DOX, MS-Zn-2-DOX and MS-Zn-3-DOX after immersion in PBS(-) at pH 7.4 and saline at pH 6.8 ( $n = 3$ ,  $*P < 0.05$ ).

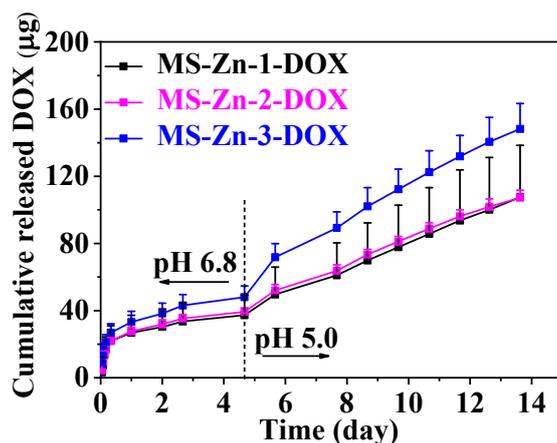


Figure S6 Amount of cumulative released DOX loaded on the MS-Zn micro-rosettes after successive immersion in the saline and acetic buffer solution ( $n = 3$ ).

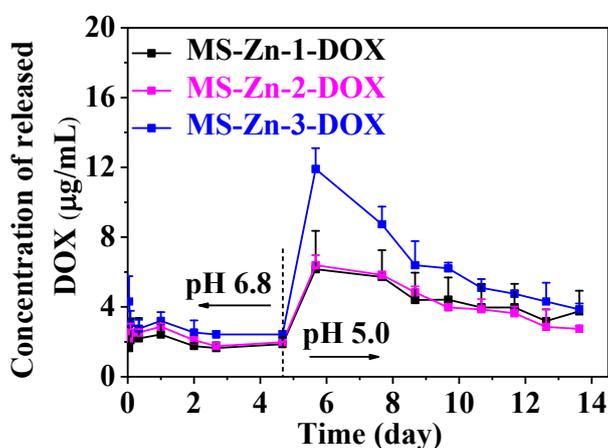


Figure S7 Concentration of released DOX after immersion MS-Zn-DOX in the saline and acetic buffer solution ( $n = 3$ ).

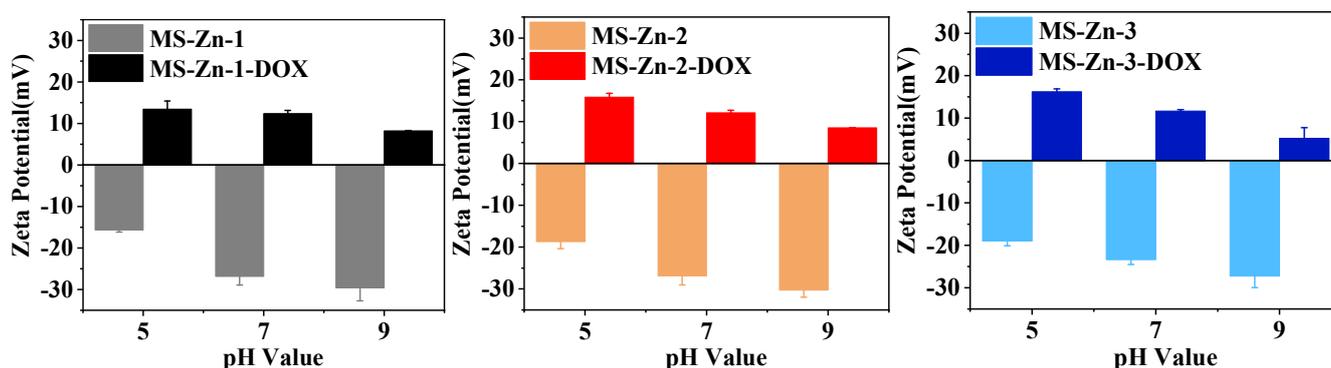


Figure S8 Zeta potentials of MS-Zn micro-rosettes before and after loaded with DOX in PBS(-) with different pH values ( $n = 3$ ).

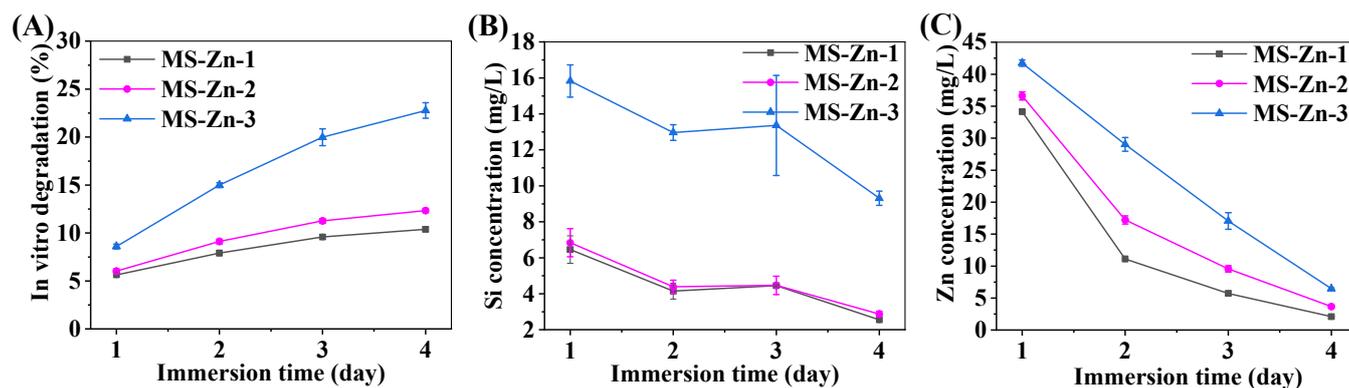


Figure S9 In vitro degradation (A), Si (B) and Zn (C) release of MS-Zn micro-rosettes after immersing in the acetic buffer solution of pH 5 for 1-4 days (n = 3).

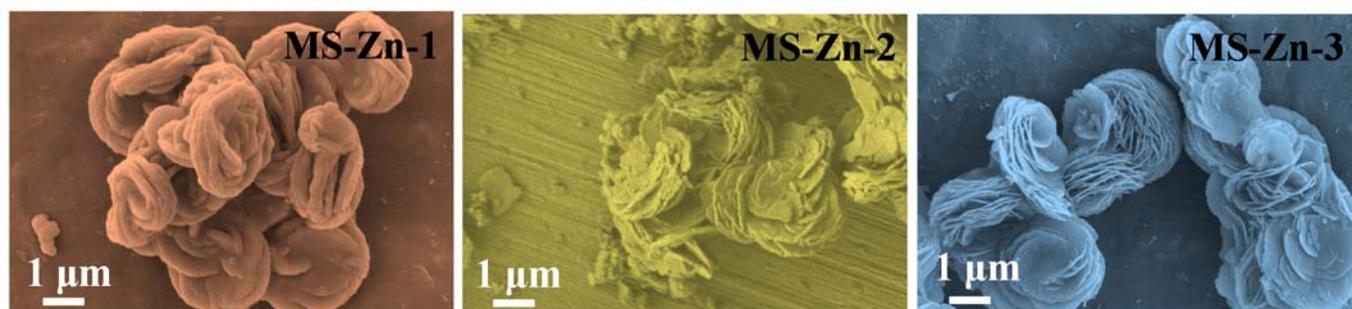


Figure S10 SEM images of MS-Zn micro-rosettes after immersing in the acetic buffer solution of pH 5 for 4 days.

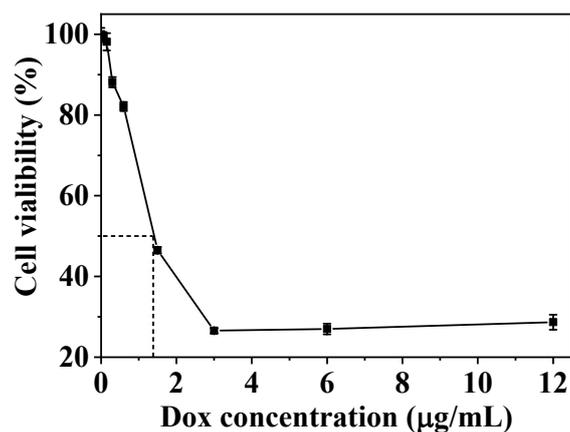


Figure S11 Cell viability of LLC cells after co-cultured with DOX (n = 8).

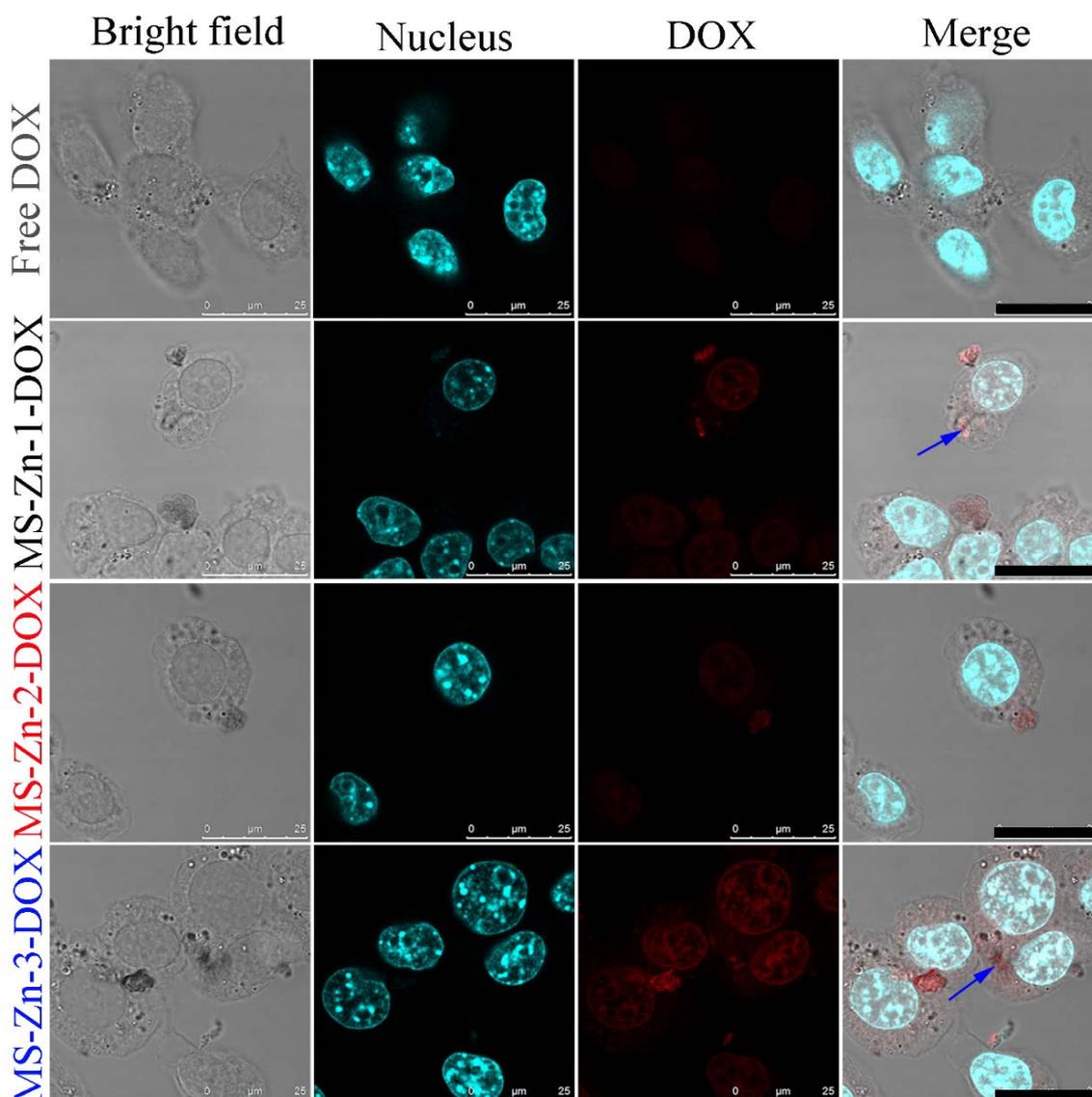


Figure S12 Representative confocal laser scanning microscopic images of LLC cells after being cultured with free DOX, MS-Zn-1-DOX, MS-Zn-2-DOX and MS-Zn-3-DOX at 37 °C for 2 h at same DOX concentration of 10 μg/mL (blue arrows indicate internalized MS-Zn-DOX, scale bar: 25 μm).

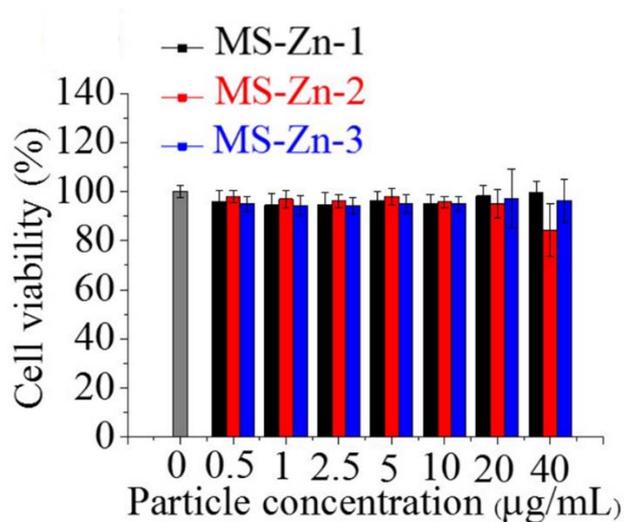


Figure S13 Viability of LLC cells after being cultured with media containing various concentrations of MS-Zn micro-rosettes (n =8).

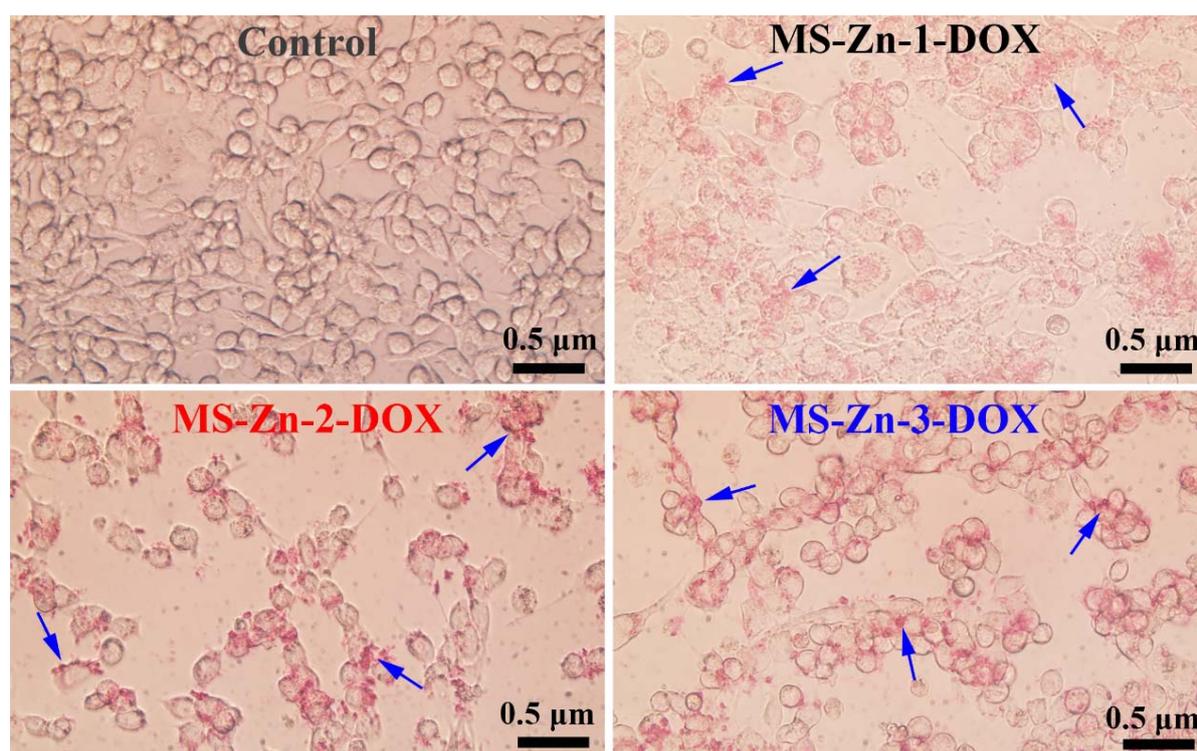


Figure S14 Representative optical microscopic images of LLC cells after being cultured with medium, or the media containing 20 µg/mL of MS-Zn-1-DOX, MS-Zn-2-DOX and MS-Zn-3-DOX at 37 °C for 24 h (the reddish particles around or inside cells were MS-Zn-DOX, as shown in blue arrows).

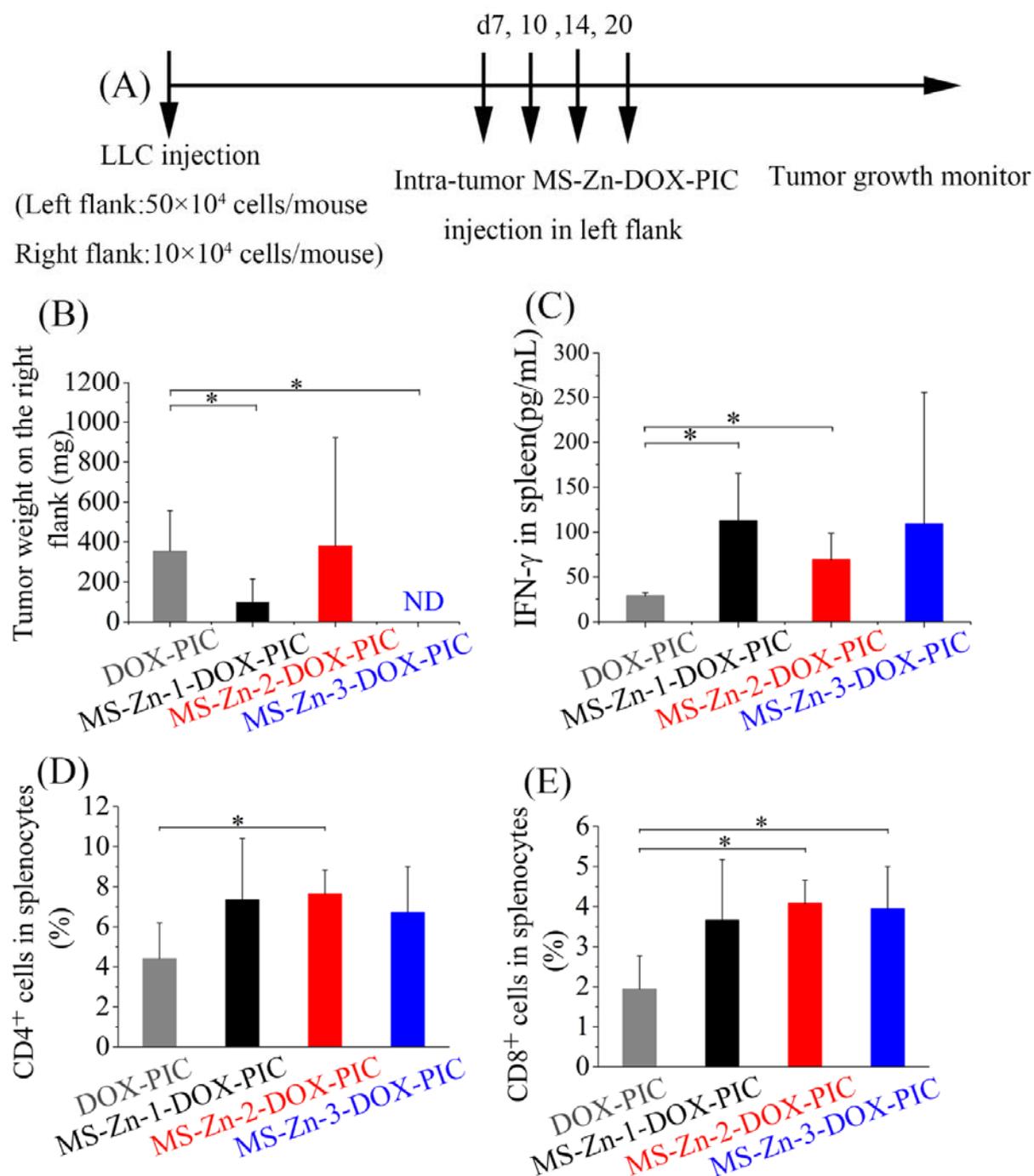


Figure S15 (A) Experimental procedure; (B) tumor weight on the right flank of mice at the end point (ND: not detected); (C) IFN- $\gamma$  cytokine secretion in spleen of mice at the end point; (D, E) summarized results of FACS. (n=5, \*P < 0.05).

## Experimental section

**Preparation of MS-Zn micro-rosettes:** In a typical method, 0.55 g of  $\text{Zn}(\text{CH}_3\text{COO})_2$  (Wako, Japan) and 10 g of N,N-dimethylethanolamine (DMEA, Wako, Japan) were dissolved in 5 mL of ultrapure water. After a homogeneous solution was obtained, different amounts of tetraethyl orthosilicate (TEOS, Wako, Japan) was added, as shown in table S1. After reacting under an ultrasonic condition at 50 °C for 10 min, the gel was hydrothermally treated in a Teflon-lined stainless steel autoclave at 160 °C for 24 h. After hydrothermal reaction, the obtained white precipitate was washed three times with ultrapure water and once with ethanol, dried at 80 °C for 6 h, crushed, and heated at 500 °C for 2 h.

**Physicochemical characterization of MS-Zn micro-rosettes:** The MS-Zn micro-rosettes were observed by a transmission electron microscopy (TEM, JEOL, Japan). The microstructure of MS-Zn was observed using a field emission scanning electron microscope (FE-SEM, JEOL, Japan) after being coated with gold. Infrared absorption bands of MS-Zn were obtained by the KBr pellet method using a Fourier-transform infrared spectrometer (FTIR, FT/IR-350, JASCO, Japan). X-ray diffractometry (XRD, RINT 2550, Rigaku, Japan) was carried out to characterize the phase of MS-Zn. Zeta potential of MS-Zn was measured using a Delta Nano C Particle Analyzer (Beckman Coulter, Inc., USA) after dispersing MS-Zn in a phosphate buffered solution (PBS(-)). PBS(-) at pH 7.4 was prepared by dissolving 4.80 g PBS powders (Dulbecco's PBS, Nissui Pharmaceutical Co., Ltd., Japan) in the 500 mL ultrapure water. The nitrogen adsorption-desorption isotherm of Zn-MS was recording using a surface area and porosity analyzer (TriStar II 3020, Micromeritics, USA) at -196 °C. The specific surface area of MS-Zn was calculated using the Brunauer–Emmett–Teller (BET) method. MS-Zn was dissolved in 1 M NaOH with subsequent addition of 2M HCl to obtain the final concentration of 1 mg/mL, and then Si/Zn molar ratio of MS-Zn was measured by an inductive coupled atomic emission spectrometer (ICP-AES, SPS7800, Seiko, Japan).

**Loading DOX onto MS-Zn micro-rosettes:** Doxorubicin hydrochloride DOX (Wako, Japan) was dissolved in PBS(-) to obtain a 2 mg/mL DOX solution. MS-Zn micro-rosettes were dispersed in PBS(-) under an ultrasonic condition to obtain a 2 mg/mL MS-Zn suspension. Then MS-Zn suspension and DOX solution were mixed at a volume of 1:1. The mixed solution was shaken at the speed of 1500 rpm for 24 h under a dark condition at room temperature. The MS-Zn micro-rosettes loaded with DOX (MS-Zn-DOX) were

collected by centrifugation followed by washing three times with PBS(-). All the supernatants were collected, and analyzed for the amount of DOX by measuring absorption intensity at 485 nm using an ultraviolet-visible (UV-vis) absorption spectrometer (V-550, JASCO, Japan). The capacity of loading DOX was defined as follows: Loading capacity (%) =  $100 \times (\text{Amount of DOX added} - \text{Amount of DOX in supernatant}) / (\text{Amount of MS-Zn added})$ .

***In vitro* DOX release experiments:** 1.0 mg of MS-Zn-DOX was putted in a regenerated cellulose tubular membrane (Nominal MWCO: 6000-8000, Membrane Filtration Products, Inc., USA). The membrane was sealed tightly using Teflon clips, and immersed in 2 mL PBS(-) at pH 7.4 or saline (Wako, Japan) at pH 6.8. The PBS(-) containing the tubular membrane were shaken under a dark condition at a speed of 40 rpm at 37 °C for different periods. As a pH-responsive release study, the membrane was immersed in an acetic buffer solution at pH 5.0 after being immersed in saline for 112 h. 75 mM acetic buffer solution at pH 5.0 was prepared by dissolving 3.014 g sodium acetate (Wako, Japan) and 1.17 mL acetic acid (Wako, Japan) in 500 mL ultrapure water. The amount of DOX released from MS-Zn micro-rosettes was determined by measuring the absorbance at 492 nm using a microplate reader (MTP-900, Corona Electric Co., Ltd., Japan).

***In vitro* degradation experiments:** The *in vitro* experiments of MS-Zn-1, MS-Zn-2 and MS-Zn-3 micro-rosettes were employed by immersion the micro-rosettes (10.0 mg) in the acetic buffer solution of pH 5.0 (10 mL) at 37 °C. At pre-determined time points, the entire buffer was collected by centrifugation, and 10 mL of the fresh buffer was added. Si and Zn ion concentrations in the collected buffer were determined by using an inductively coupled plasma-atomic emission spectrometry (ICP-AES, Hitachi High-Technologies). The degradation ratio of the micro-rosettes was calculated according to the Si and Zn concentrations. After immersion for 4 days, the micro-rosettes were observed by a field-emission scanning electron microscope (FE-SEM, JEOL, Japan) after being coated with gold.

**Cellular uptake of MS-Zn-DOX:** Uptake of MS-Zn-1-DOX, MS-Zn-2-DOX, MS-Zn-3-DOX and free DOX by the Lewis lung carcinoma (LLC) cells was observed using a confocal laser scanning microscope (CLSM,

Leica Confocal IP/FCS, German), and quantitatively determined using a microplate reader (MTP 900, Corona Electric Co., Ltd., Japan). In detail,  $4 \times 10^4$  LLC cells were seeded in a cell culture dish 38 mm in diameter and cultured with the medium containing 90 vol.% low glucose dulbecco's modified eagle's medium (L-DMEM, Wako, Japan) and 10 vol.% fetal bovine serum (FBS, Invitrogen, USA) at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. After overnight culture, the medium was changed into those containing free DOX, MS-Zn-1-DOX, MS-Zn-2-DOX or MS-Zn-3-DOX, followed by culture for another 2 h. All groups contained the same amount of DOX (10 µg/mL). Then the cells were washed two times with PBS(-) gently, fixed using 4 vol.% paraformaldehyde with incubation on ice for 20-30 min, and finally washed three times with PBS(-). The cell nucleus was stained with 1 µg/mL Hoechst 33258 in PBS(-) with incubation at 37 °C for 15 min. The fluorescent images were obtained using a confocal laser scanning microscope at the excitation wavelength of 351 and 488 nm for Hoechst 33258 and DOX, respectively. For quantitative analysis,  $4 \times 10^4$  LLC cells were seeded in each well of a 96-well plate. After overnight culture, the medium was changed into 100 µL fresh one containing MS-Zn-1-DOX, MS-Zn-2-DOX or MS-Zn-3-DOX, or free DOX. All groups contained the same amount of DOX (10 µg/mL) by adjusting particle concentration. After 1, 2 and 3 h culture, the medium was removed, and the cells were washed three times with PBS(-) gently. Subsequently, the fluorescent intensity was measured using a microplate reader (MTP-900, Corona Electric Co., Ltd., Japan) at excitation and emission wavelength of 492 nm and 663 nm, respectively.

**Cytotoxicity evaluation:** Cytotoxicity of MS-Zn micro-rosettes loaded and unloaded with DOX was determined using LLC cells with CCK-8 assay.  $4 \times 10^4$  LLC cells were seeded in each well of a 96-well plate. After overnight culture, the medium containing 90 vol.% L-DMEM and 10 vol.% FBS was changed into 100 µL of fresh one containing different amounts of MS-Zn micro-rosettes loaded and unloaded with DOX. After 24 h culture, the medium was changed into 150 µL of fresh one containing 10 vol.% CCK-8 working solution. After incubation at 37 °C for 1 h under a dark condition, 100 µL of supernatant was transferred into each well of a 96-well plate followed by measuring absorbance at 450 nm using a microplate reader (680 XR, BIO-RAD, USA). The viability of cells was normalized to those without any particles.

**Immune response test by macrophages-like cells:** *In vitro* immunogenic activity of MS-Zn micro-rosettes was evaluated by measuring the amount of interleukin-1 $\beta$  (IL-1 $\beta$ ) and TNF- $\alpha$  secreted by macrophage-like cells differentiated from human monocytic leukemia cells (THP-1: Riken Bio Resource Center, Japan). At first,  $6 \times 10^7$  THP-1 cells were cultured in a condition medium containing 90 vol.% RPMI 1640 (Invitrogen, USA), 10 vol.% FBS and 0.5  $\mu$ M phorbol 12-myristate 13-acetate (PMA, Sigma, USA) in a 75 cm<sup>2</sup> flask for 3 h. The macrophage-like cells were washed three times with the medium containing 90 vol.% RPMI 1640 and 10 vol.% FBS, and suspended at a concentration of  $2 \times 10^6$  cells/mL in the medium containing 90 vol.% RPMI 1640 and 10 vol.% FBS. Next, 100  $\mu$ L of the previous macrophage-like cell suspension was seeded in each well of a 96-well plate. After overnight culture, the medium in each well was changed into those containing 10  $\mu$ g/mL of MS-Zn-1, MS-Zn-2 or MS-Zn-3 micro-rosettes. After 24 h culture, amounts of IL-1 $\beta$  and TNF- $\alpha$  in the media were measured using human IL-1 $\beta$  and TNF- $\alpha$  ELISA kits (BD Pharmingen, USA) according to the manufacturer's instructions.

***In vivo* anti-tumor effect:** The animal experiment was approved by the Ethical Committee of the National Institute of Advanced Industrial Science and Technology (AIST). All animal experiments and feeding were carried out in accordance with the guidelines of the Ethical Committee of AIST, Japan. As shown in figure S15 A, live LLC cells were subcutaneously injected into the left flank ( $5 \times 10^5$  cells/mouse) and the right flank ( $1 \times 10^5$  cells/mouse) of mice (C57BL/6J, female, 6 weeks old, CLEA Inc., Japan) at day 0. A mixture of PIC at 1 mg/mL, DOX at 1 mg/mL and one of either MS-Zn-1, MS-Zn-2 or MS-Zn-3 at 10 mg/mL in 100  $\mu$ L PBS(-) was injected into the tumor on the left flank of mice at 7, 10, 14 and 20 d, respectively. The groups injected with only PBS(-), and a mixture of free DOX and PIC in PBS(-) were used as the controls. Tumor diameters on the left flank and right flank were measured using a caliper at predetermined time points. At day 27, tumor formed on the right flanks were collected from the mice and weighted.

**Analysis of isolated spleen in mice:** At day 27, spleens were excised for flow cytometry and enzyme-linked immunosorbent assay (ELISA). For flow cytometry, the spleen was mashed gently in the RPMI 1640 medium containing 2 vol.% FBS. The single-cell suspension was obtained after incubation with red blood cell lysing buffer (Sigma-Aldrich, USA) for 5 min on ice, washing with 0.5% FBS contained PBS(-) for

three times. Anti-CD16/CD32 antibody (2.4G2, Biolegend) was used to block non-specific staining. Anti-mouse CD4 and anti-mouse CD8 $\alpha$  (Biolegend) were used to stain the cells after incubation at 4 °C for 30 min under a dark condition. Flow cytometry was performed using FACSAria (BD Bioscience, USA). For ELISA, the spleen was digested with a tissue protein extraction reagent (Thermo Scientific, USA), and analyzed for IFN- $\gamma$  using ELISA kits (BD Bioscience, USA) according to the manufacturer's instructions.

**Statistical analysis:** In this study, all data presented as mean value  $\pm$  standard deviation (SD). Student's t-test and Log-Rank test were used to analyze differences between experimental groups. A value of  $p < 0.05$  was regarded as statistical significance.

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