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Electronic Supplementary Information

pH-responsive gold nanoparticles-in-liposome hybrid nanostructures for enhanced systemic tumor delivery

Jutaek Nam,[a] Yeong Su Ha,[b] Sekyu Hwang,[a] Woonghee Lee,[b] Jaejung Song,[c] Jeongsoo Yoo,[b] Sungjee Kim*[ac]

[a] *Department of Chemistry, Pohang University of Science & Technology (POSTECH), San 31, Hyojadong, Namgu, Pohang 790-784, South Korea, Fax: +82-279-1498; Tel:+82-279-2108; E-mail: Sungjee@postech.ac.kr*

[b] *Department of Molecular Medicine, School of Medicine, Kyungpook National University, Dongin-dong, Joong-gu, Daegu 700-422, South Korea*

[c] *School of Interdisciplinary Bioscience and Bioengineering, POSTECH, South Korea*

Experimental details

Materials and characterization. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DOPE-PEG) was purchased from Avanti Polar Lipids. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS), and other reagents were obtained from Sigma. All chemicals were used as received without further purification. Lipid solutions were prepared in chloroform and stored at - 20 °C. Water was triply distilled using Millipore filtration system. UV-Vis absorption spectra were obtained using Agilent 8453 UV–Vis spectrophotometer and fluorescence spectra were measured on Jobin Yvon Horiba SPEX FluoroLog 3 spectrofluorometer. Hydrodynamic size and zeta potential were measured using Malvern Zetasizer Nano S and

Zetasizer Nano Z, respectively. TEM images were recorded using JEOL JEM-2100.

Preparation of SAN-encapsulating PEG-grafted liposome (SANLIPO). pH-responsive 'smart' gold nanoparticle (SAN) was synthesized as described in the previous report.[S1] They were encapsulated in liposomal vehicles by following a reverse phase evaporation method with slight modifications.[S2] Briefly, 10 mg/ml chloroform solutions of DOPE (450 μl, 6 μmol), CHEMS (200 μl, 4 μmol), and DOPE-PEG (85 μl, 0.3 μmol) were placed and mixed in a glass vial, and the solvent was removed under reduced pressure using vacuum pump. The lipids were re-dissolved in a solvent mixture of 100 μl chloroform and 200 μl diethyl ether, in which 1 μM SAN phosphate buffered saline (PBS) solution (100 μl) was added. The resulting two-phase system was sonicated for 1 h in a bath-type sonicator (Branson 3510) to form homogeneous emulsion. The mixture was then placed on the rotary evaporator and the organic solvent was removed under reduced pressure at room temperature. The SAN-encapsulating PEG-grafted liposome (SANLIPO) was separated from free SANs by continuous density gradient centrifugation.[S3] Water/glycerol mixture with different compositions (30, 40, and 50 vol% glycerol) was prepared and placed on the bottom of each other (400 μl) in the e-tube, starting with the 30% mixture at the top. The crude product of SANLIPO (200 – 600 μl) was layered on top of the density gradient medium and centrifuged at 18,000 g for 1 h. SANLIPO was remained at the upper layer while free SANs completely settled down to the bottom. SANLIPO was carefully extracted from the e-tube and further purified from glycerol by spin down at 2,300 g for 1 h followed by re-dispersion in PBS.

Calcein release assay. Calcein was encapsulated in liposomes at a self-quenched concentration of 100 mM and the leakage of calcein from the liposomes was monitored by the increase of fluorescence intensity.[S2b, S4] Calcein-loaded liposomes was placed in 10 mM

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pH 7.4 phosphate buffer or 10 mM pH 5.5 acetate buffer at 37 °C, and time evolution of calcein fluorescence was measured for each solution. To correct pH effect on the calcein fluorescence, free calcein was also placed in the same buffer condition and the fluorescence intensity was identically measured over time. Calcein fluorescence after 100% leakage was simulated by adding 0.1% Triton X-100 to the SANLIPO solution. The percentage of calcein leakage was calculated from the expression, $(I_t - I_0)/(I_{100} - I_0)$, where I_t and I_0 is the fluorescence intensity measured at time t and 0, respectively, and I_{100} is the totally recovered calcein fluorescence intensity by Triton X-100 treatment. I_t and I_{100} were corrected by accounting the fluorescence intensity changes of free calcein under same treatment conditions.

Cellular internalization and accumulation of SAN and SANLIPO. B16 F10 mouse melanoma cells were purchased from the Korean Cell Line Bank. B16 F10 cells were maintained in minimum essential medium with Earle's balanced salts (HyClon), which was supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Gibco). B16 F10 cells were grown onto 12 mm glass coverslips in 24-well plates at a density of 1×10^5 cells/well at 37 °C under 5% CO_2. After 1 day, the cells were either treated with 5 nM of pristine SAN or SANLIPO containing 5 nM of SAN. They were co-incubated for 24 h at 37 °C under 5% CO_2, and then cells were rinsed with PBS three times and fixed with 4% formaldehyde at room temperature for 20 min. The cells were further washed with PBS three times. Cellular internalization and accumulation of SAN and SANLIPO were monitored by dark-field microscopy using Zeiss Axioplan 2 microscope with a high numerical dark-field condenser (0.75 – 1.0) and a 100× / 1.3 oil iris objective lens. Images were taken using Zeiss Axiocam HR camera.

Biodistribution of SANLIPO in tumor-bearing mice. Six-week-old female C57BL/6 mice

(16 – 18 g body weight) were purchased from Hyochang Science (Daegu, South Korea). The mice were anesthetized by inhalation of 1 – 2% isoflurane, and then B16 F10 cells (1×10^7 cells/ml) were injected subcutaneously into the pre-shaved area of the right flank. The mice were randomly sorted for treatments when the tumors reached a diameter of ~1 cm. They were anesthetized, and 100 μl PBS solutions of SANLIPO with 200 nM of encapsulated SANs was administered intravenously via the tail vein. They were allowed to circulate for 1, 4, 24, 48, or 72 h (n=3 for each case). Stomach, heart, lung, spleen, liver, kidney, and tumor tissues were excised after the mice were sacrificed at each time point, which were followed by lysis using aqua regia. The determination of the gold concentration in each organ and tumor tissue was performed quantitatively by inductively coupled plasma atomic emission spectrometry (Thermo Electron Co.). All animal experiments were conducted in compliance with the Animal Care and Use Committee requirements of Kyungpook National University.

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Supplementary Figures

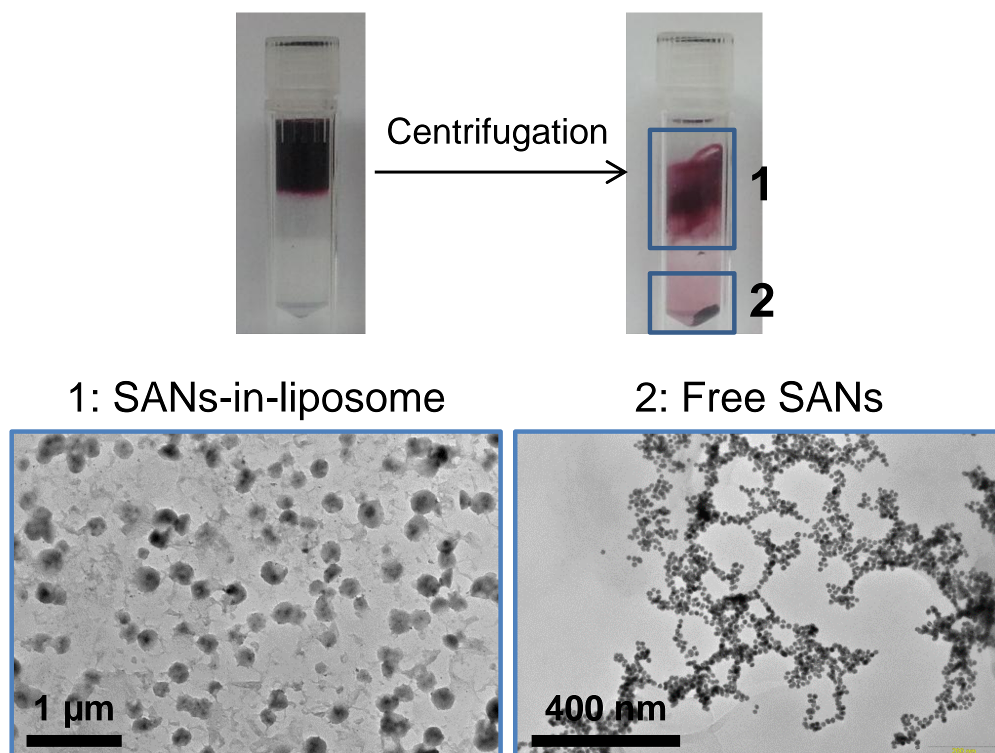

Fig. S1 Density gradient centrifugation of the as-prepared SANLIPO using layered water/glycerol mixture medium with different compositions (30, 40, and 50 vol % glycerol). Pictures showing the consequence of centrifugation at 18,000 g for 1 h (top) and corresponding TEM images measured for different portions (bottom). SANLIPO was remained at the upper layer (portion **1**), while free SAN completely settled down to the bottom (portion **2**). TEM for SANLIPO (SANs-in-liposome) was measured after 2% uranyl acetate staining.

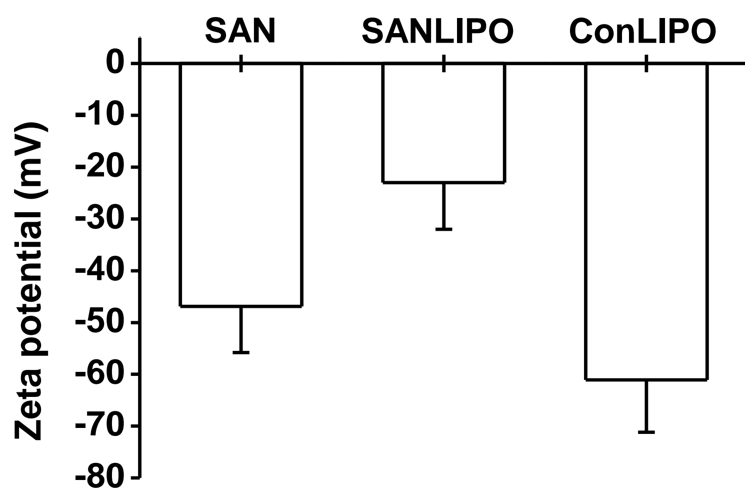

Fig. S2 Zeta potential of SAN, SANLIPO, and control liposome prepared without DOPE-PEG (ConLIPO).

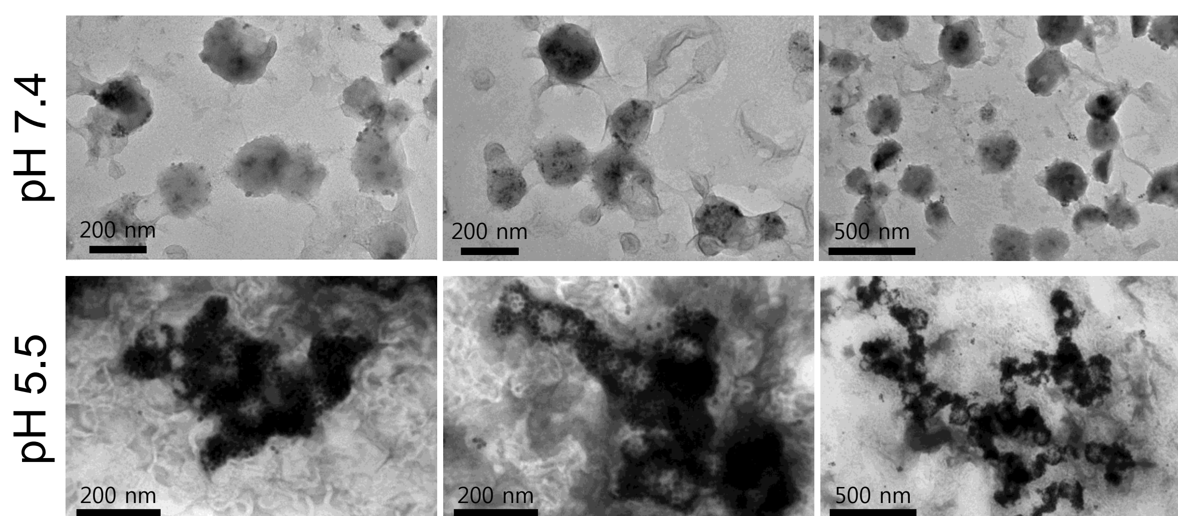

Fig. S3 TEM images of SANLIPO after 180 min in pH 7.4 (top) or in pH 5.5 (bottom) measured after 2% uranyl acetate staining.

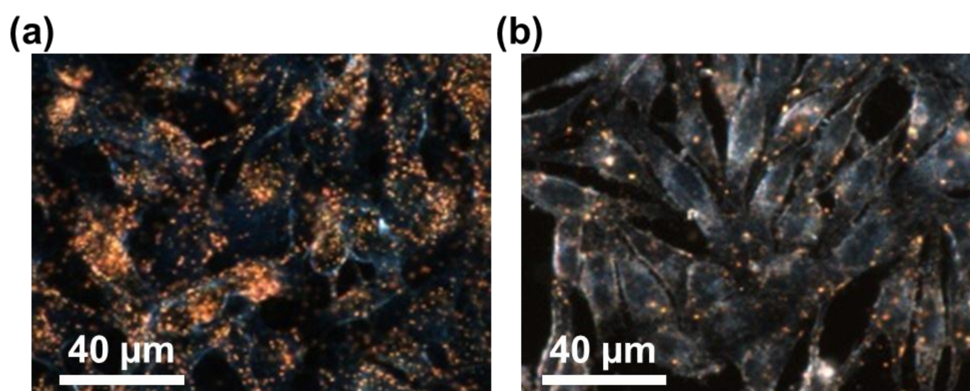

Fig. S4 Dark-field microscope images of B16 F10 cells incubated for 24 h with 5 nM of pristine SAN (a) or SANLIPO containing 5 nM of SAN (b).

References

S1. J. Nam, N. Won, H. Jin, H. Chung and S. Kim, *J. Am. Chem. Soc.*, 2009, **131**, 13639.

S2. (a) F. Szoka and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, 1978, **75**, 4194; (b) V. A. Slepushkin, S. Simões, P. Dazin, M. S. Newman, L. S. Guo, M. C. P. de Lima and N. Düzgüneş, *J. Biol. Chem.*, 1997, **272**, 2382.

S3. D. Steinigeweg, M. Schütz, M. Salehi and S. Schlücker, *Small*, 2011, **7**, 2443.

S4. G. R. Bartlett, *J. Biol. Chem.*, 1959, **234**, 466.