Supporting Information

Dual Secured Nano-Melittin for Safe and Effective Eradicating Cancer Cells

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Materials:

Glycol chitosan (GCS, Mw = 200 kDa; degree of deacetylation = 90%), potassium persulfate, sodium borohydride, succinic anhydride, melittin from bee venom, sodium dodecyl sulfate, D-(+)-trehalose dehydrate, N-succinimidyl 3-[2-pyridyldithio]-propionate (SPDP), tris(2-carboxyethyl)phosphine (TCEP), triethlyl amine (TEA), ethylenediaminetetraacetic acid (EDTA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). L-cysteine HCl and Ellman's reagent were purchased from Thermo Scientific (Rockford, IL, USA). Dulbecco's Modified Eagle Medium (DMEM) supplied with high glucose, trypsin and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from ATCC (Manassas, VA, USA).

Depolymerization of glycol chitosan

Glycol chitosan (Mw: 200 kDa) was first depolymerized to Mw 28 kDa using potassium persulfate, and then amidized and thiolated by reacting with succinic anhydride and SPDP sequentially. Briefly; glycol chitosan (400 mg) was dissolved in 37.5 ml of hydrochloric acid (2%, v/v). After that, potassium persulfate (67.5 mg) was added to the above glycol chitosan solution to start the depolymerization. The reaction was kept at 70 °C for 2 h and then stopped by adding sodium borohydride (300 mg). The reaction mixture was neutralized with sodium

hydroxide (1 M). The resulting polymer was dialyzed (Spectra 7 MWCO: 3500 Da) against ddH_2O for 24 h followed lyophilization. Molecular weight of the polymer was obtained from GPC (viscotek GPCmax VE 2001 GPC solvent/sample module, viscotek VE 3580 RI detector and 270 Dual Detector) using 0.3 M acetic acid, 0.3 M sodium acetate, 0.02% sodium azide, 1% ethylene glycol (pH 4.5) as the mobile phase.

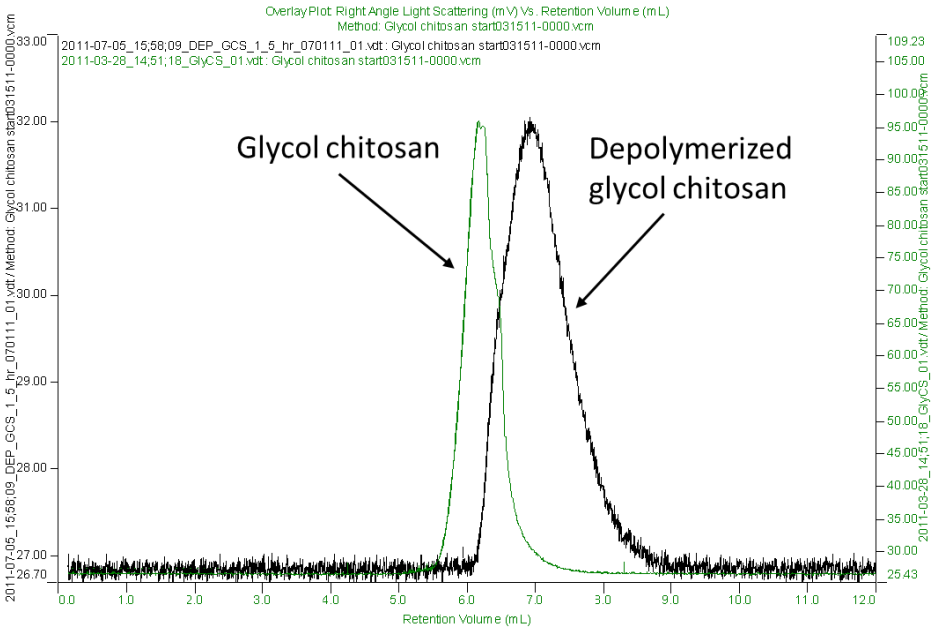

Fig. S1 The GPC spectra of glycol chitosan and depolymerized glycol chitosan.

Synthesis of succinic anhydride modified glycol chitosan (SA-GCS)

Succinic anhydride (SA) was grafted onto the above yielded low molecular weight glycol chitosan (28 kDa) by amidation. Briefly; glycol chitosan (30 mg) was dissolved in deionized water (6 mL) and succinic anhydride (4.17 mg) was added under stirring. After 2 h of reaction at room temperature, the pH of the reaction mixture was adjusted to 8 with NaOH (1 M). The reaction was then allowed to proceed for another 2 h. The product was purified by dialysis (Spectra 7 MWCO: 3500 Da) against water (pH 9) for one day and lyophilized for 48 h. The structural composition of amidized glycol chitosan (SA-GCS) was characterized by ^{1}H-NMR

spectroscopy. The appearance of methylene proton peaks at δ = 2.4 to 2.6 ppm corresponding to succinic acid along with the glycol chitosan peaks at δ = 2.06, 2.75, 3.7 and 4.5 ppm (Fig. S2). To determine the IEP of SA-GCS, ζ potential was measured as a function of pH using Zetasizer Nano-ZS (Malvern, UK). In a typical experiment, 0.5 mg/mL polymer solution was prepared in NaCl (10 mM), and the surface charges of SA-GCS at different pHs were recorded.

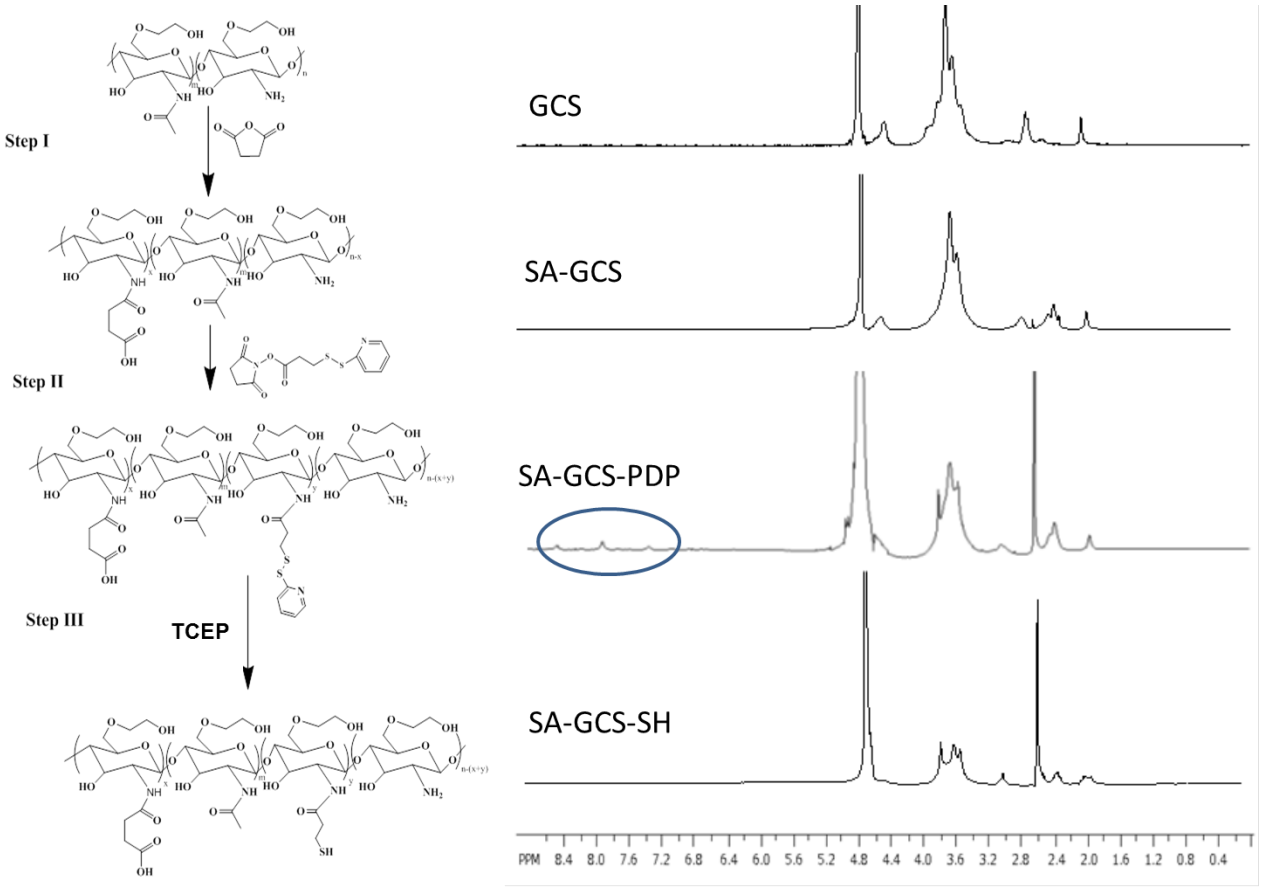

Fig. S2 Scheme for the synthesis of SA-GCS, SA-GCS-PDP, and SA-GCS-SH and their corresponding ^{1}H NMR spectra in D_2O. The circled area indicates the peaks of PDP.

Synthesis of thiolated amidized glycol chitosan (SA-GCS-SH)

To generate thiol groups on SA-GCS, SA-GCS was reacted with SPDP. The introduced disulfide bonds were then cleaved by TCEP (Fig. S2) to yield free thiol groups. Briefly; SA-GCS

(60 mg) was first dissolved in dimethyl sulfoxide (10 mL). TEA (300 µL) and SPDP (21.63 mg) were then added under stirring. After overnight reaction at room temperature, the disulfide bonds were cleaved by adding TCEP thereby generating free thiol group. Then, the polymer was purified by dialysis (Spectra 7 MWCO: 3,500 Da) against 10 mM EDTA (24 h) followed by ddH_2O (24 h). The product was obtained after lyophilization for 48 h and stored at -20°C. IEP of SA-GCS-SH was determined in a similar way as SA-GCS. The concentration of thiol group was quantified by DTNB assay. The number of thiol groups per polymer molecule was found to be 8.7 as calculated by DTNB assay.

Nano-complexes preparation and characterization

Melittin and SA-GCS-SH were dissolved in Tris buffer saline (TBS) pH 7.4 to get 0.1 mg/mL and 5 mg/mL, respectively. Then, 1 ml melittin solution was incubated with different amounts (0.1 to 4 mL) of SA-GCS-SH solution for 1 h at room temperature to get non-crosslinked complexes (single secured nano-sting, SSNS). To develop cross-linked complexes (dual secured nano-sting, DSNS), SSNS was aerially oxidized for 2 h at room temperature. SSNS and DSNS were freeze-dried with 5% trehalose and stored at -20 °C until use. The binding efficiency of SA-GCS-SH was measured by measuring the fluorescence of free melittin. Free melittin from the complexes was separated by centrifugal filtration at 14,000 rcf. for 8 min. using Nanosep 30 K Omega (Pall corporation, USA) and the concentration was determined by measuring its fluorescence (λ_{EX}=280nm, λ_{EM}=350 nm) using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices). Nano-complexes prepared at the ratio of 200:1 (polymer:melittin, w/w) were used for the following experiments.

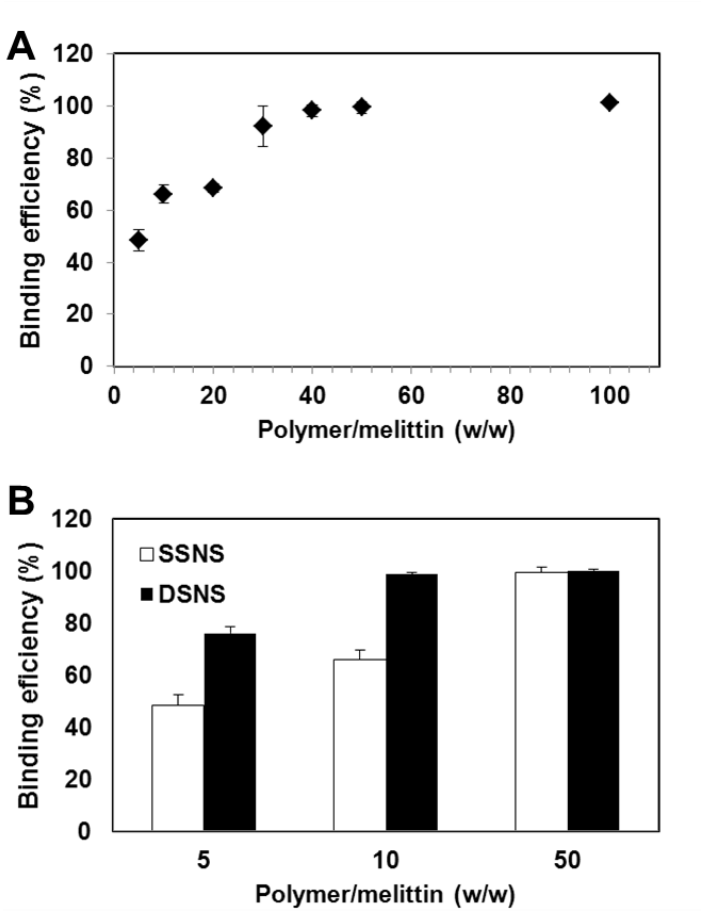

Fig. S3 Binding efficiency **c**haracterization of nano-complexes. Binding efficiency of nano-complex formed at different polymer/melittin (w/w) ratios (A), Binding efficiency of SSNS and DSNS at different polymer/melittin (w/w) ratios (B). Data represents mean ± SD, n=3.

The size and surface charge (ξ-potential) of the complexes were measured using a Zetasizer Nano-ZS (Malvern, UK) at pH 7.4. The morphology of the complexes was observed by transmission electron microscopy (Hitachi H-800 TEM) using a formvar/carbon coated Copper Grids (Electron Microscopy Science). The original complexes solution (5 μL) was dropped on a grid, washed with ddH_2O (3 ×) to remove the remaining salt, and dried with a tissue paper.

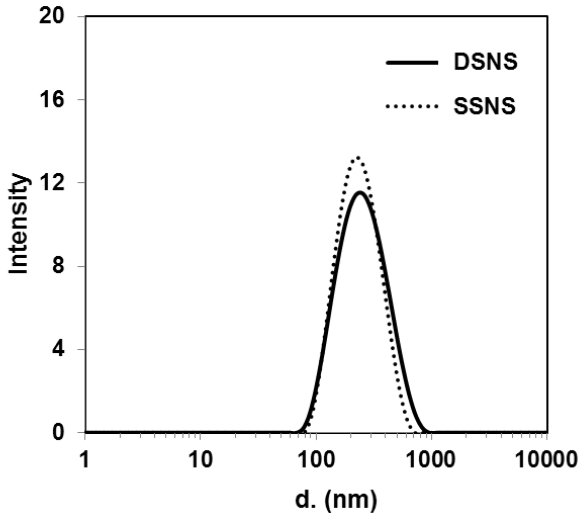

Fig. S4 The size distribution of SSNS and DSNS determined by dynamic light scattering.

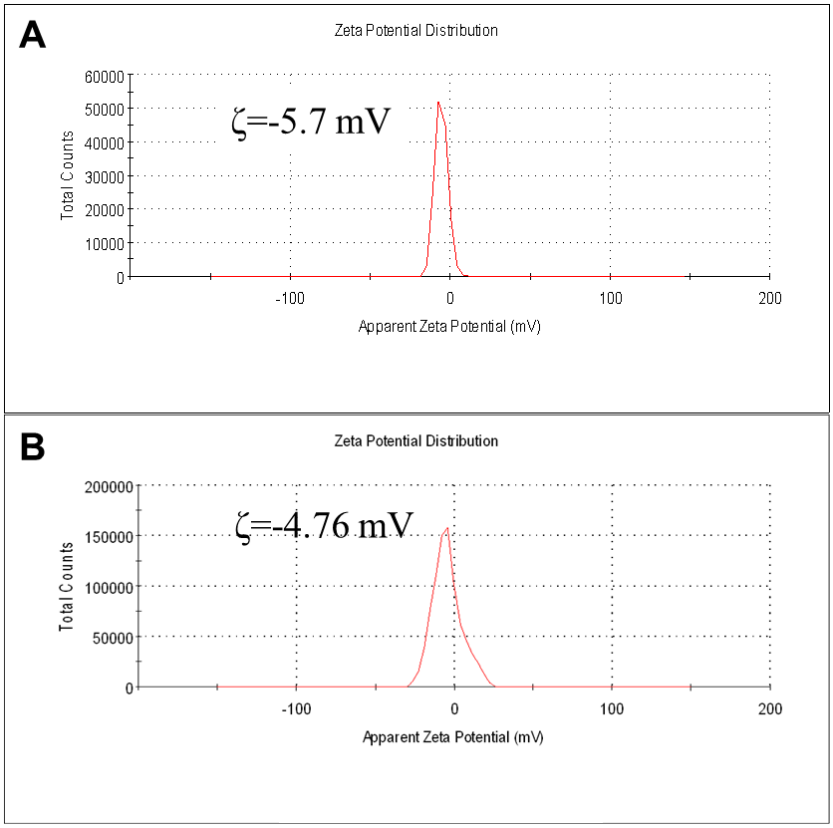

Fig. S5 The surface charge of SSNS (A) and DSNS (B) determined by phase analysis light scattering.

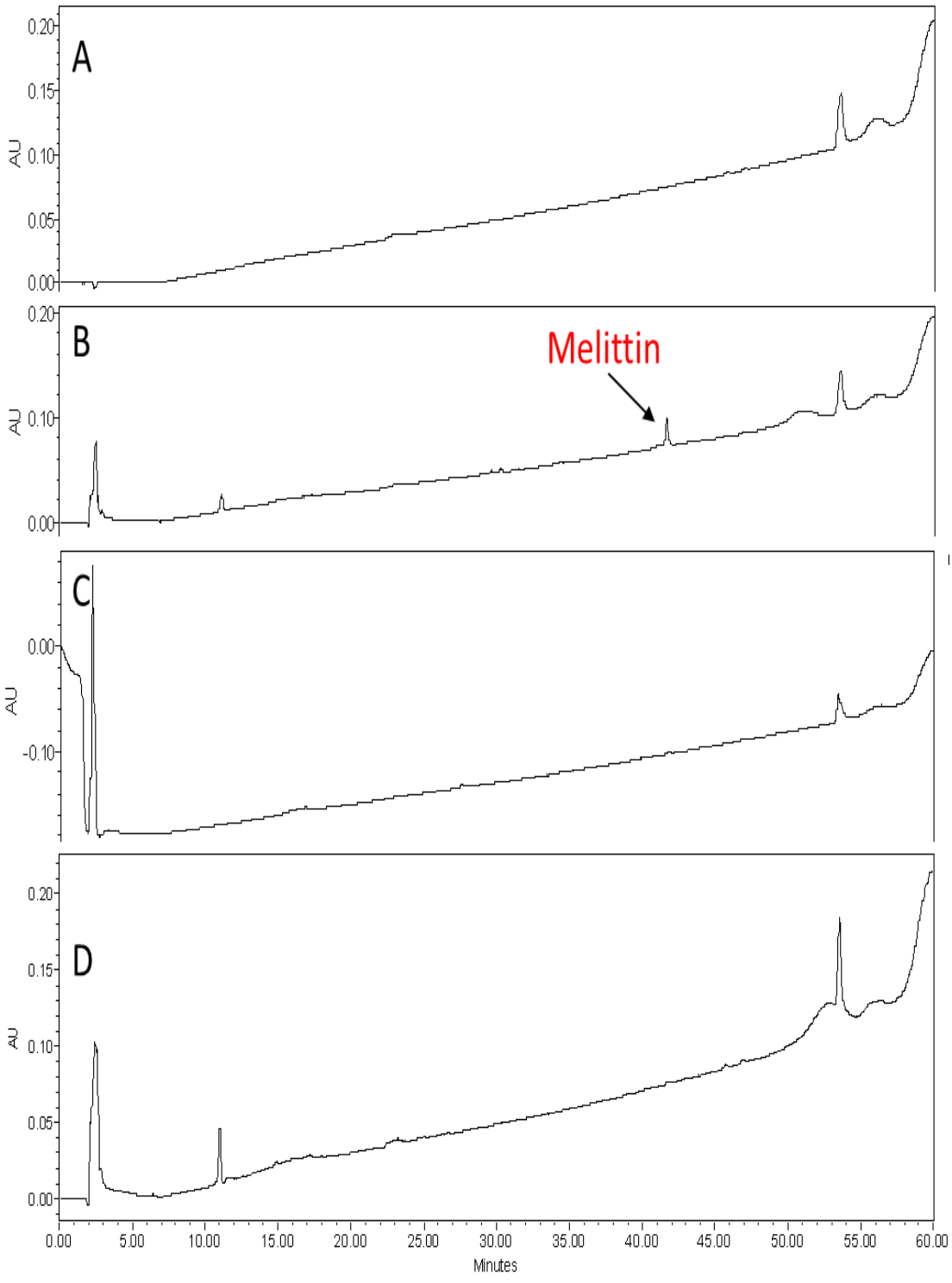

Fig. S6 HPLC spectra of SSNS and DSNS for the detection of free melittin in the nano-complexes. (A) Solvent, (B) free melittin, (C) SSNS, and (D) DSNS.

HPLC determination of melittin was carried out with a Waters 2996 instrument equipped with a photodiode array detector and a hypersil gold column (250 × 4.6 mm, Fisher); mobile phase: mixture of acetonitrile and water supplemented with 0.1 % TFA (linear gradient from 4:96 to 64:36, v/v, over 60 min); flow rate: 1.5 ml/min; detection: 220 nm. The retention time for melittin was 41.6 min. Standard solutions were prepared by dissolving melittin in ddH_2O followed by a series of dilutions. Melittin stock solution was prepared at 100 μg/mL. For sample preparation, 50 μg of SSNS and DSNS were dissolved in 500 μl ddH_2O and the free melittin solution was collected by centrifugation at 13,000 rcf for 30 min twice with Nanosep centrifugal device 10K Omega (Paul life sciences). The resulting filtrate (50 μL) was injected for HPLC determination. No peak detected at 41.6 min suggested that both SSNS and DSNS were free of free melittin (Fig. S6).

FRET measurement

The donor fluorescence dye Cy3-NHS was chemically conjugated with SA-GCS and SA-GCS-SH prior to forming the complex. Similarly, the receptor fluorescence dye Sulfo-Cy5-NHS was conjugated with melittin. The conjugated Cy3 amounts in SA-GCS and SA-GCS-SH were adjusted carefully to ensure that the same conjugation efficiency was achieved, which was further validated by measuring the fluorescence intensity of Cy3 with a SpectraMax M5 Multi-Mode Microplate Reader. After that, SSNS was fabricated from Cy3-labeled SA-GCS and Cy5-labeled melittin, while DSNS was fabricated from Cy3-labeled SA-GCS-SH and Cy5-labeled melittin. Cy3 and Cy5 dual-labeled SSNS and DSNS were prepared following the same protocol except that the DSNS needed aerial oxidation. Therefore, the amount of each dye in SSNS and DSNS was the same. The titration of nano-complex was carried out by adding 0.1 M HCl and

NaOH to adjust pH to the pre-designed values. Both SSNS and DSNS samples were loaded into Coring® 96 well black flat bottom plates. Samples were excited at 500 nm with the cutting off 530 nm. The entire fluorescence spectra (from 530 nm to 750 nm) of both nano-complexes were then recorded as a function of the pH with the help of a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices).

Release kinetics of SSNS and DSNS

Cy5 labeled melittin were fabricated into SSNS and DSNS as described above. SSNS and DSNS samples (2 mL) were loaded into dialysis bags (MWCO: 6000-8000). The dialysis bags were put into different media (pH 7.4,10 mM PBS and pH 5, 10 mM acetic acid buffer) at 37 °C under continually stirring. At predesigned time (1, 2, 4, 8 h), 1 mL sample was taken and supplemented with 1 mL corresponding fresh medium. Samples were lyophilized and re-suspend in 200 μL ddH_2O. To calculate the release amount of melittin, the originally labeled Cy5-melittn was employed to obtain standard curve with the help of a fluorescence microplate reader. The fluorescence intensities of released samples were quantified under excitation of 630 nm and emission of 670 nm.

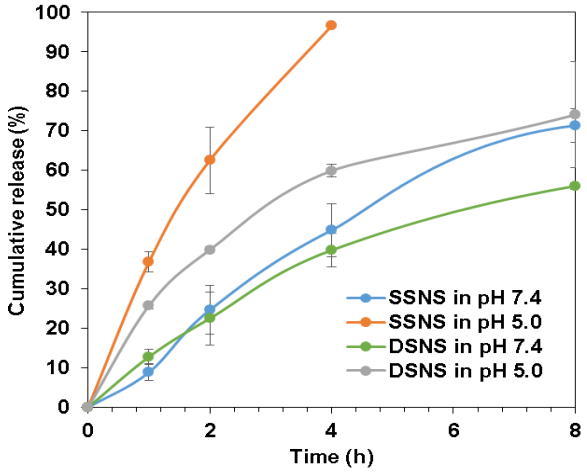

Fig. S7 The release kinetics of SSNS and DSNS at pH 7.4 and 5.0 buffers.

Hemolytic assay

Sprague Dawley Rat whole blood was purchased from bioreclamation LLC (NC, USA). Briefly, RBCs were washed with NaCl (210 mM) until no red color was visible in the supernatant. The washed RBCs were used to compare hemolytic effects of melittin, SSNS and DSNS using DI water as positive control and PBS (pH 7.4) as negative control. Melittin of different concentrations (0.1 to 5 μM) and SSNS or DSNS (equivalent with free melittin) were added to fixed number of washed RBCs (2.5×10^7 cells) and incubated at 37 ^{o}C for 1 hour. The release of hemoglobin was quantified by measuring the absorbance at 405 nm of the supernatant in a microplate reader (EL_X808, Bio-Tech Instrument) after centrifugation at 300 rcf. for 2 minutes. The results were expressed relative to the reference standard. To investigate the effect of pH and redox potential on the hemolytic activity of SSNS and DSNS, nano-complexes were co-incubated with RBCs in PBS (pH 5.0) and PBS (pH 7.4) supplemented with 10 mM GSH, respectively. The stability of DNSN was also tested by co-incubating RBCs with DSNS in PBS buffer containing 50% serum.

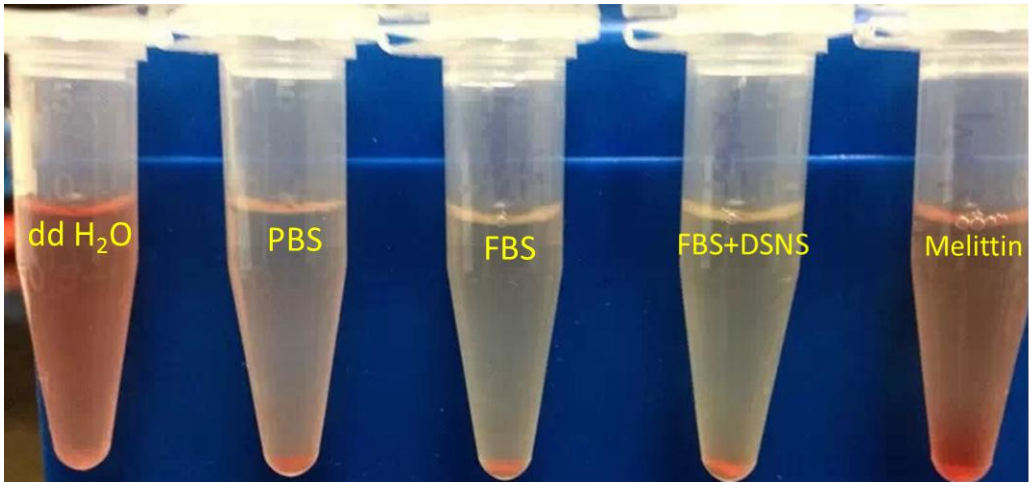

Fig. S8 The image of RBCs after co-incubating with DSNS in 50% serum containing buffer.

Confocal microscopy

HCT-116 cells were seeded at a density of 2×10^4 cells per chamber slides and allowed to grow for 24 h. SA-GCS and SA-GCS-SH were conjugated with Cy3 followed by excessive dialysis to remove free Cy3. The purified Cy3 labeled polymer were used to prepare SSNS and DSNS. After treated with SSNS and DSNS for 2 h at 37°C in a 5% CO_2 incubator, cells were washed with PBS three times and fixed with 4% formaldehyde in PBS, followed by staining with Hoechst 33342 1 μg/mL (Invitrogen) and washed again with PBS for 3 times. Finally, cells were imaged with confocal laser scanning microscopy (Zeiss LSM 700).

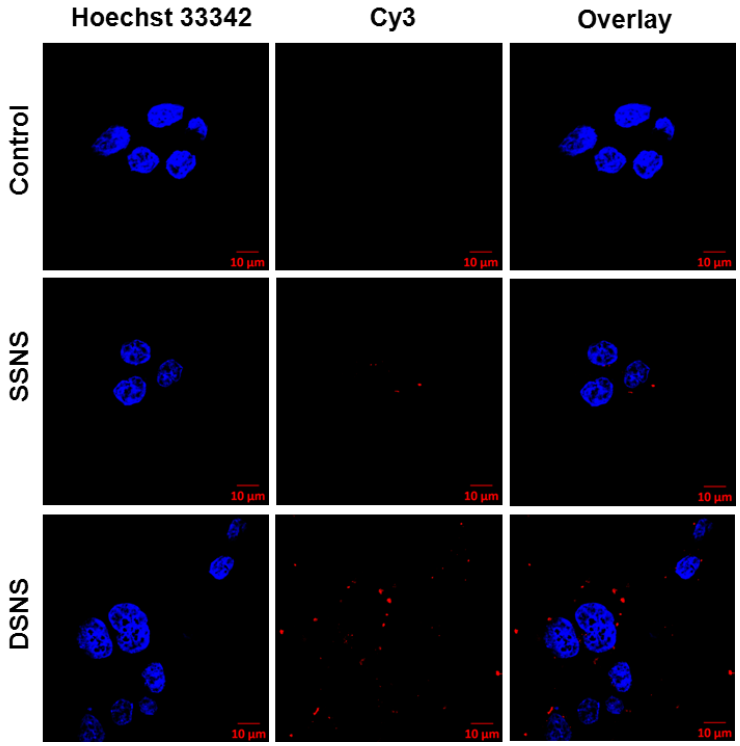

Fig. S9 Confocal microscope images of HCT-116 colon cancer cells after incubation with SSNS and DSNS for 2 h. Scale bars are 10 μm in all images.

JC-1 Staining

HCT 116 cells were seeded in each petri dish at density of 300,000 cells per dish. After overnight incubation, cells were treated with 2 µM Melittin or DSNS for 1 h. Cells were then stained with JC-1 for 30 min (Cayman chemical, MI), and observed under confocal microscope using FITC and Texas Red channels.

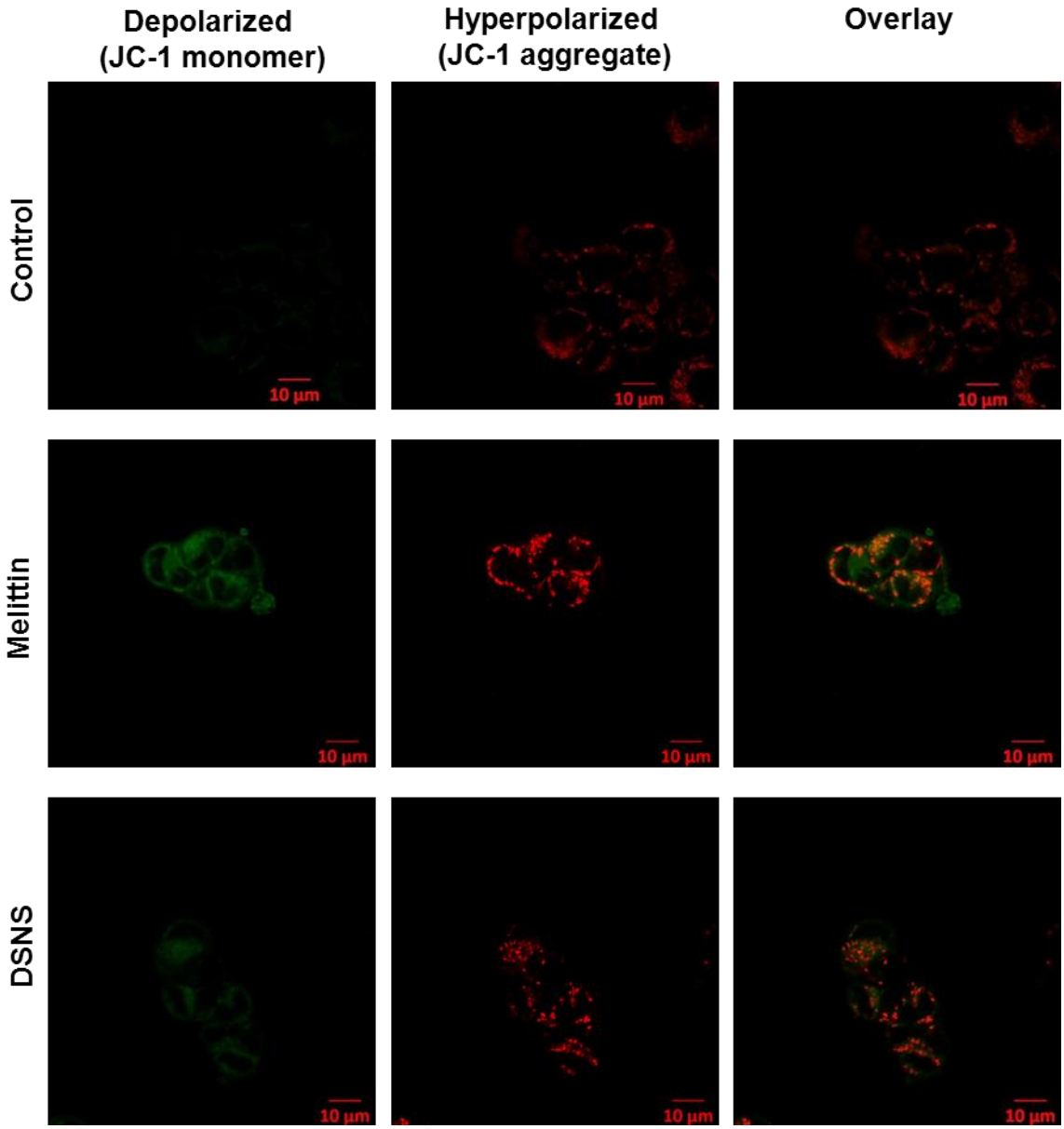

Fig. S10 Confocal microscope images of HCT-116 colon cancer cells after incubation with melittin and DSNS for 1 h and stained with JC-1. Scale bars are 10 μm in all images. Green fluorescence indicates depolarized JC-1 (monomer form) due to the damaged mitochondria function, and red indicates hyperpolarized JC-1 (aggregation).

Cytotoxicity assay

The anticancer activities of melittin and DSNS in MCF-7, HCT-116, SKOV-3 and NCI/ADR-res cells were evaluated by MTT assay using melittin as a positive control and non-treated cells as a negative control. The cells were seeded in 96-well plates at an initial density of 12,000 cells/well in 150 μl of DMEM medium supplemented with 100 U penicillin/streptomycin and 10% FBS. After 24 h, the medium was replaced with 150 μL of fresh medium containing DSNS and melittin (corresponding to 0.1 to 10 μM melittin) and incubated for another 24 h. After the defined time of co-incubation, the media were replaced with 100 μL fresh media containing 1 mg/mL MTT reagent and incubated for 4 h. The formed MTT crystal was dissolved with a stop solution and the finally optical density of the medium was measured using a microplate reader (ELX808, Bio-Tech Instrument, Inc) at λ = 595 nm. The cytotoxicity of melittin and DSNS was calculated as relative to the control group (untreated cell). The cytotoxicity of oxidized SA-GCS-SH polymer was evaluated in parallel.

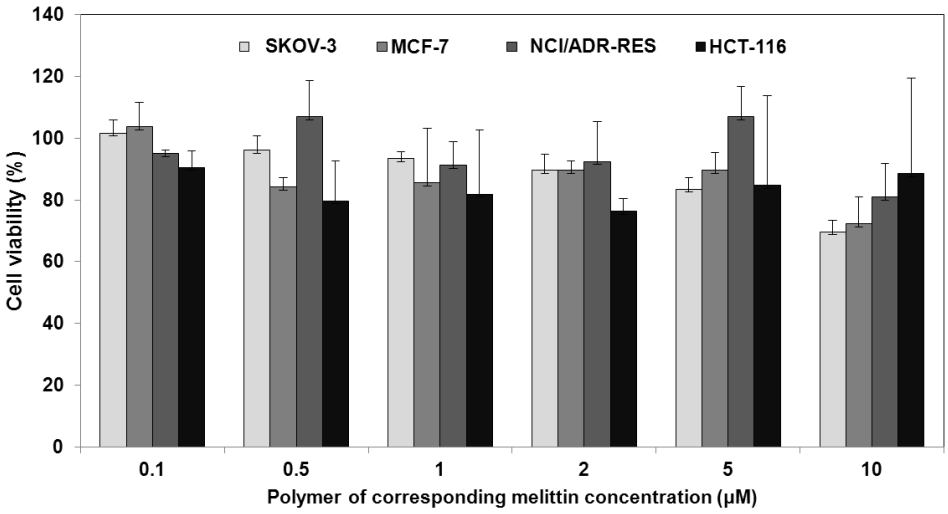

Fig. S11 Cytotoxicity of SA-GCS-SH polymer at the corresponding melittin concentration of 0.1 to 10 μM for SKOV-3, MCF-7, NCI/ADR-RES, and HCT-116 cancer cells.