Self-assembled Hybrid Nanoparticles for Targeted Co-delivery of Two Drugs into Cancer Cells

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Supporting Information

Materials and Methods

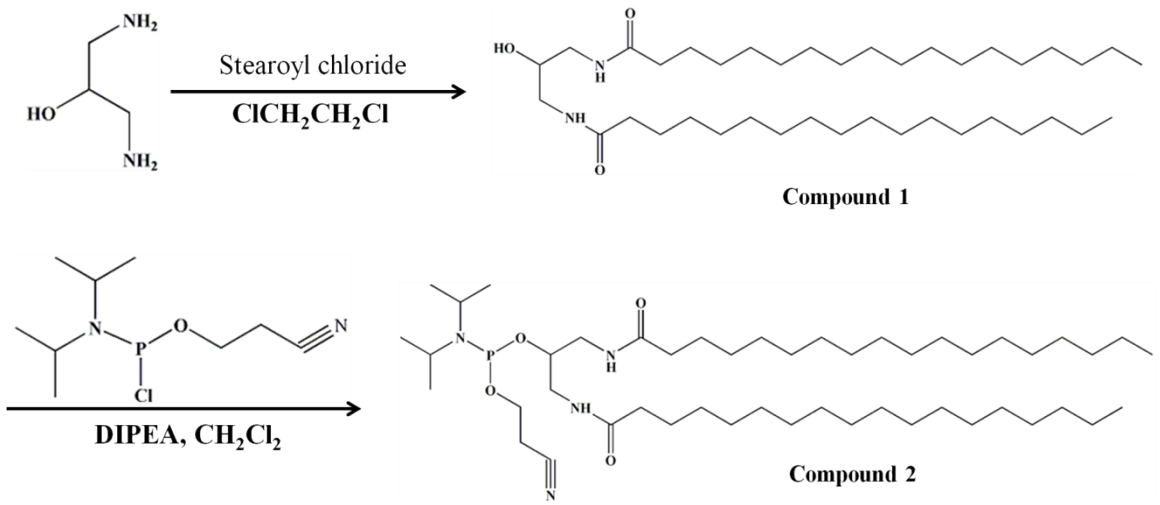
Materials. The materials for DNA synthesis were purchased from Glen Research (Sterling, VA). All reagents for buffer preparation and HPLC purification came from Fisher Scientific. Unless otherwise stated, all chemicals were used without further purification. Lecithin, paclitaxel (PTX), doxorubicin hydrochloride (DOX) and PLGA with 1/1 lactide/glycolide monomer ratio were obtained from Sigma-Aldrich. DSPE-PEG2000 was purchased from Avanti. TRITC-DHPE (N-(teramethylrhodamine-6-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt) was purchased from Invitrogen.

(1) Synthesis of lipid phosphoramidite.

Synthesis of compound 1 (see Scheme S1 for structures): A solution of stearoyl chloride (5.00 g) in 50 mL of 1,2-dichloroethane was added dropwise to a solution of 1,3-diamino-2-hydroxypropane (0.73 g) and triethylamine (TEA) (2.57 mL) in 100 mL of 1,2-dichloroethane. The reaction mixture was stirred under a blanket of argon at room temperature for 2 h and then at 70 °C overnight. The mixture was then cooled to room temperature. The solid product was filtered, washed with CH_2Cl_2, CH_3OH, 5% $NaHCO_3$ and $CH_3CH_2OCH_2CH_3$ in order, and vacuum dried to yield compound 1 as a white solid. The product was identified by ^1H NMR according to data reported in the literature. [1]

Synthesis of compound 2: N, N-diisopropylethylamine (DIPEA) (4.19 mL) was injected into a solution of compound 1 (3.00 g). The solution was cooled on an ice bath under a blanket of argon, and

then 2-cyanoethyl-N, N-diisopropylchlorophosphoramidite (2.15 mL) was added dropwise. The reaction mixture was stirred at room temperature for 1 h and then at 80 °C for 1.5 h. The solution was then cooled to room temperature, washed with 5% $NaHCO_3$ and brine, Na_2SO_4 dried, and vacuum concentrated. The product was precipitated as a white solid by adding the concentrated solution to CH_3CN. The product was identified by ^1H NMR and ^{31}P NMR according to data reported in the literature. [1]



Scheme S1. Detailed synthesis route for lipid phosphoramidite.

(2) Synthesis of oligonucleotide probes

All oligonucleotide probes were synthesized on an automated ABI 3400 DNA synthesizer (Applied Biosystems, Foster City, CA, USA) by solid phase oligonucleotide synthesis starting from the corresponding controlled pore glass (CPG) beads. Lipid phosphoramidite was dissolved in CH_2Cl_2 and then coupled onto the oligonucleotide sequences. Oligonucleotide probes containing carboxytetramethylrhodamine (TAMRA) were deprotected in 3 mL TAMRA deprotection solution (methanol : tert-butylamine : water = 1: 1 : 2) at 65 °C for 4 h, and all others were deprotected in 3 mL AMA solution (ammonium hydroxide : 40% aqueous methylamine = 1 : 1) at 65 °C for 30 min. All oligonucleotide probes were precipitated by adding 250 μL of 3 M NaCl and 6 mL of cold CH_3CH_2OH.

Then the precipitated oligonucleotide probes were collected by centrifugation at 4000 rpm for 30 min and dissolved in 400 μL of triethylammonium acetate (TEAA) for further purification by reversed-phase high-pressure liquid chromatography (HPLC) (ProStar, Varian, Walnut Creek, CA, USA). Oligonucleotide probes with and without diacyllipid were purified using C4 and C18 columns, respectively, using CH_3CN-TEAA solution as the mobile phase. Finally, these oligonucleotide probes were quantified by measuring their absorbances at 260 nm using a Varian Cary 100 UV-Vis spectrometer (Agilent Technologies, Santa Clara, CA, USA). The DNA sequences are shown in Table S1.

Table S1: Detailed sequence information for all oligonucleotide probes

Name	**Sequence**
Sgc8-tail1	5'-ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA TTTTTCGACGACGACGACGACGACGA-TAMRA-3'
Sgc8-tail2	5'-ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA TTTTTCGACGACGACGACGACGACGA-3'
Lipid-DNA	5'-lipid-$(PEG)_8$-TCGTCGTCGTCGTCGTCGTCG-FAM-3'

The red regions in the above table correspond to Sgc8 aptamer. Underline denotes base pairs of DOX loading sites.

(3) Synthesis of nanoparticles

The nanoparticles were synthesized as previously described [2] using Lipid–PEG–DNA triblock ligands. A 3-mL DSPE-PEG/Lipid-PEG-DNA/lecithin mixture in 4% ethanol containing 0.17 mg DSPE-PEG /Lipid-PEG-DNA (1:4 molar ratio) and 0.08 mg lecithin was heated for 3 min above the lipid phase transition temperature (68 °C) with gentle stirring. After heating, 1 mg of PLGA with 0.1 mg PTX in 1 mL acetone was added dropwise at 1 mL/min. The solution was vortexed vigorously for

3 min, followed by self-assembly under gentle stirring for 2 h at RT. The NPs were washed three times using an Amicon Ultra-4 centrifugal filter with 100,000 Da molecular weight cutoff (Millipore). The NPs were resuspended in pH 7.2 PBS buffer and 2 mM EDTA and incubated with 1 μM DOX for 30 min at RT. The NPs were again washed three times using an Amicon Ultra-4 centrifugal filter with 30,000 Da molecular weight cutoff (Millipore) to remove the free doxorubicin.

(4) Characterization of Nanoparticles

TEM grids were prepared by adding NP samples (1 mg/mL) in H_2O onto copper grids. Samples were blotted after 10 min, and the grids were negatively stained for 10 min at RT with freshly prepared, sterile-filtered, 3 wt% uranyl acetate solution. The uranyl acetate solution was blotted away, and the grids were air dried before imaging. DLS measurements were performed on a ZetaPALS DLS detector (Brookhaven Instruments, Holtsville, NY, USA) at 25 °C, using a 3 mL disposable cuvette. The scattering angle was fixed at 90°. The steady-state fluorescence measurements were recorded on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon, Edison, NJ) with a 0.2 cm quartz cell, using a band-pass of 5 nm for both excitation and emission. To ensure that the PTX had been encapsulated into the nanoparticles, 0.1 mL of the nanoparticle solution (1mg/mL) was mixed with an equal volume of acetonitrile to dissolve the NPs. PTX content was quantified by HPLC (ProStar, Varian, Walnut Creek, CA, USA) at a retention time of ~12–16 min in a 1 mL/min, 1/1 acetonitrile/water, isochratic mobile phase. PTX absorbance was measured at 227 nm using a UV-Vis detector. Finally, the amount of PTX retained was calculated on the basis of the peak area.

(5) Cell culture and binding of nanoparticles with cells

CCRF-CEM (CCL-119, T-cell line, human ALL) and Ramos (CRL-1596, B-cell line, human Burkitt's lymphoma) were cultured in RPMI 1640 medium (American Type Culture Collection) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 0.5 mg/mL penicillin streptomycin

(American Type Culture Collection) at 37 ºC under a 5% CO_2 atmosphere. Cells were washed with washing buffer (4.5 g/L glucose and 5 mM $MgCl_2$ in Dulbecco's PBS) and resuspended in binding buffer (0.1 mg/mL yeast tRNA and 1 mg/mL BSA in washing buffer). Cells and nanoparticles (labeled with FAM and TAMRA) were incubated on ice for 30 min. After washing with 1.0 mL washing buffer, the cells were either tested by a FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) by counting 20 000 events, or subjected to confocal microscope imaging using an Olympus FV500-IX81 confocal microscope (Olympus, Center Valley, PA, USA).

(6) Internalization of nanoparticles

Cells were incubated with nanoparticles (labeled with FAM or TAMRA) at 37 ºC for 2 h. They were then washed with 1 mL of washing buffer to remove the free nanoparticles and suspended in binding buffer for confocal imaging.

(7) Cytotoxicity test

The cytotoxicity study was performed using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS) for CCRF-CEM and Ramos cell lines in a 96-well cell culture plate at 200k cells/well. First, the cells were allowed to bind with four types of nanoparticles (NPs only, NPs with DOX, NPs with PTX, NPs with DOX and PTX) for 2 h at 37 ºC, followed by centrifugation at 1260 rpm for 3 min to remove the free NPs. Cells were then incubated at 37 ºC under 5% CO_2 for 48 h, during which cells grew in log phase. Finally, a 6x diluted MTS solution (120 μL/well) in RPMI-1640 medium solution was added to each well and incubated at 37 ºC for 2 h. The absorbance value at 490 nm was determined by a VersaMax microplate reader (Molecular Devices, Inc.).

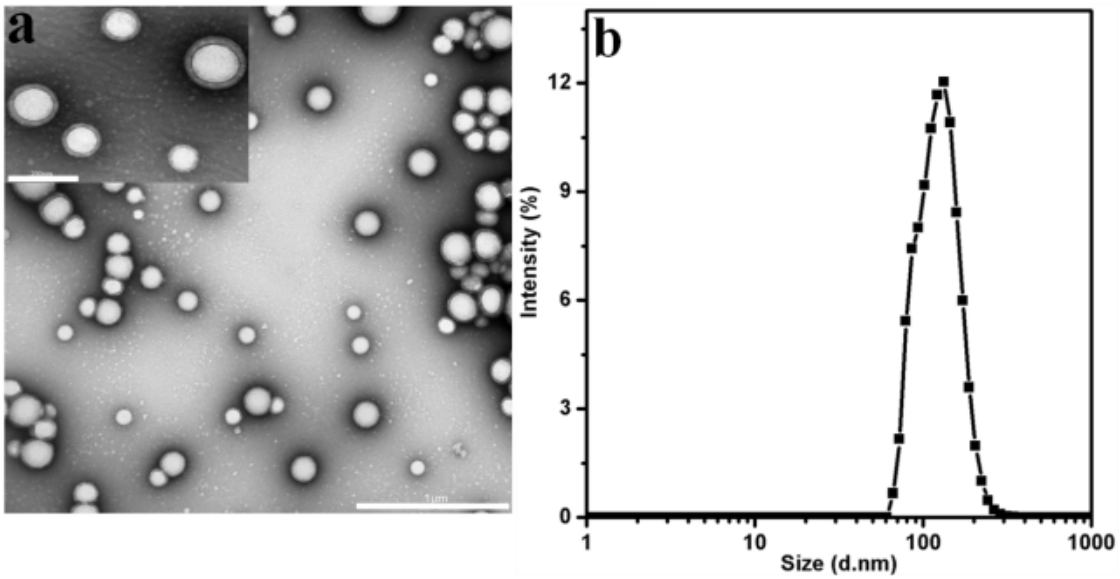


Fig. S1 Characterization of nanoparticles by (a) TEM and (b) DLS measurements. The scale bars of the TEM images represent 1μm or 200 nm (inset).

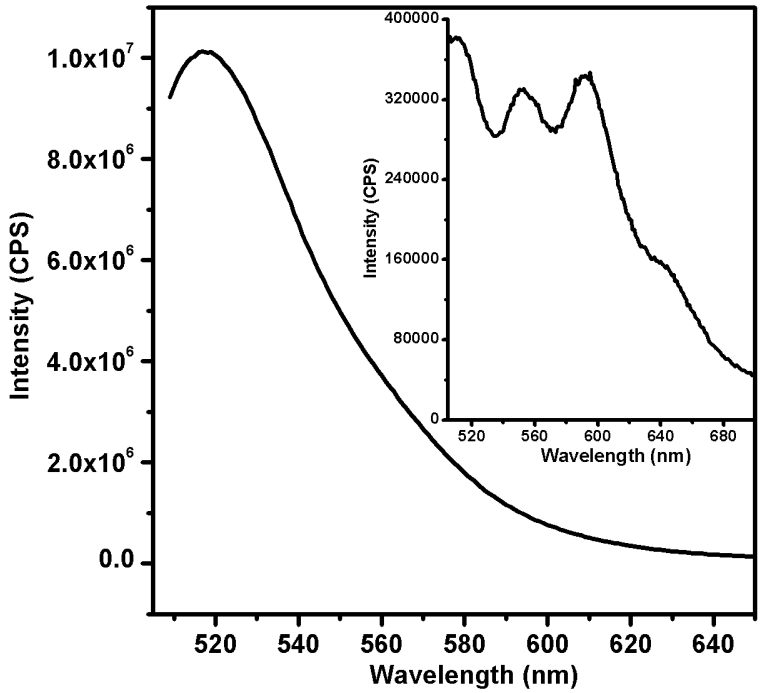


Fig. S2 Fluorescence spectrum of FAM-labeled NPs and NPs with DOX (inset). The results show that DOX had been successfully loaded into the NPs.

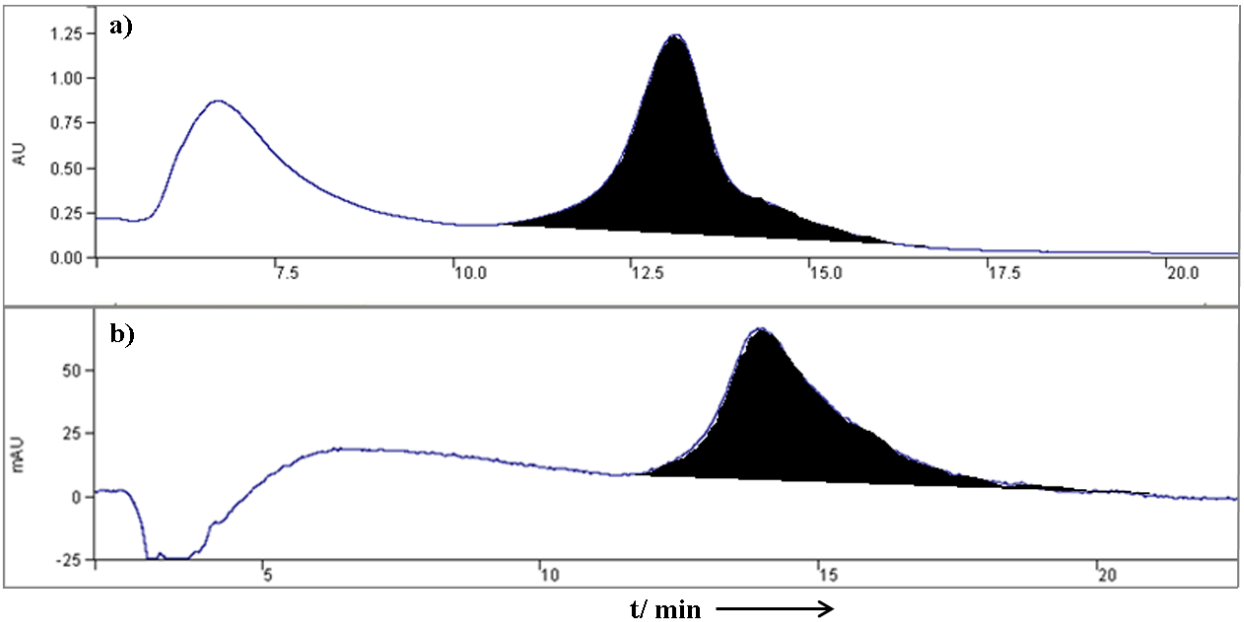


Fig. S3 HPLC measurements of (a) 100 μg free PTX dissolved in acetonitrile and (b) 100 μg NPs with PTX dissolved in acetonitrile. The results show that PTX had been encapsulated into the NPs. The peak area was analyzed by NIH ImageJ software.

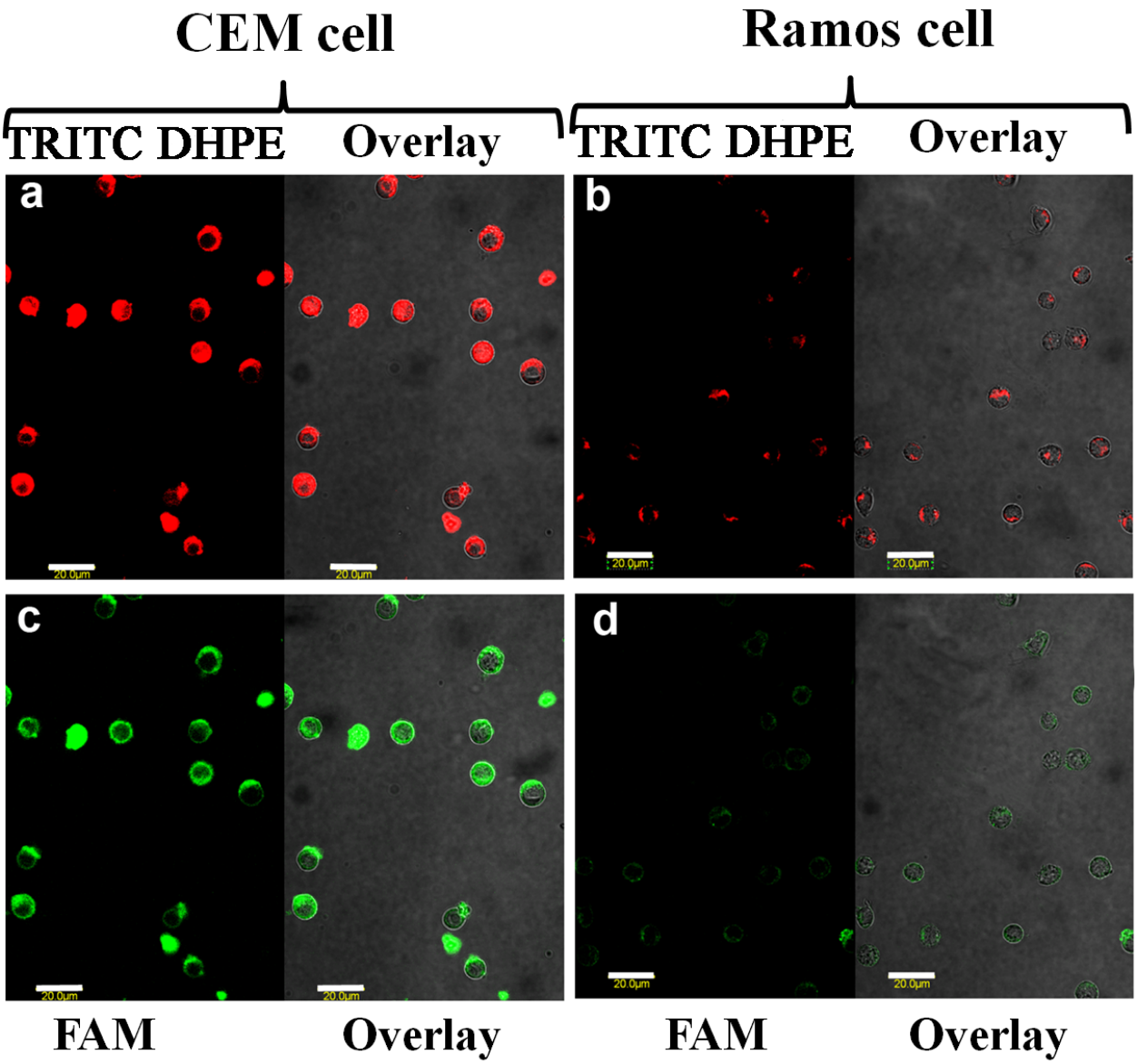


Fig. S4 Confocal laser scanning microscopy images of CEM and Ramos cells treated with 10 μg FAM-labeled nanoparticles encapsulating TRITC DHPE for 2 h at 37 °C.

References:

[1] Liu, H. P.; Zhu, Z.; Kang, H. Z.; Wu, Y. R.; Sefan, K.; Tan, W. H., *Chem-Eur J* **2010**, 16, 3791-3797.

[2] Chan, J. M.; Zhang, L. F.; Tong, R.; Ghosh, D.; Gao, W. W.; Liao, G.; Yuet, K. P.; Gray, D.; Rhee, J. W.; Cheng, J. J.; Golomb, G.; Libby, P.; Langer, R.; Farokhzad, O. C., *P. Natl. Acad. Sci. USA* **2010**, 107, 2213-2218.