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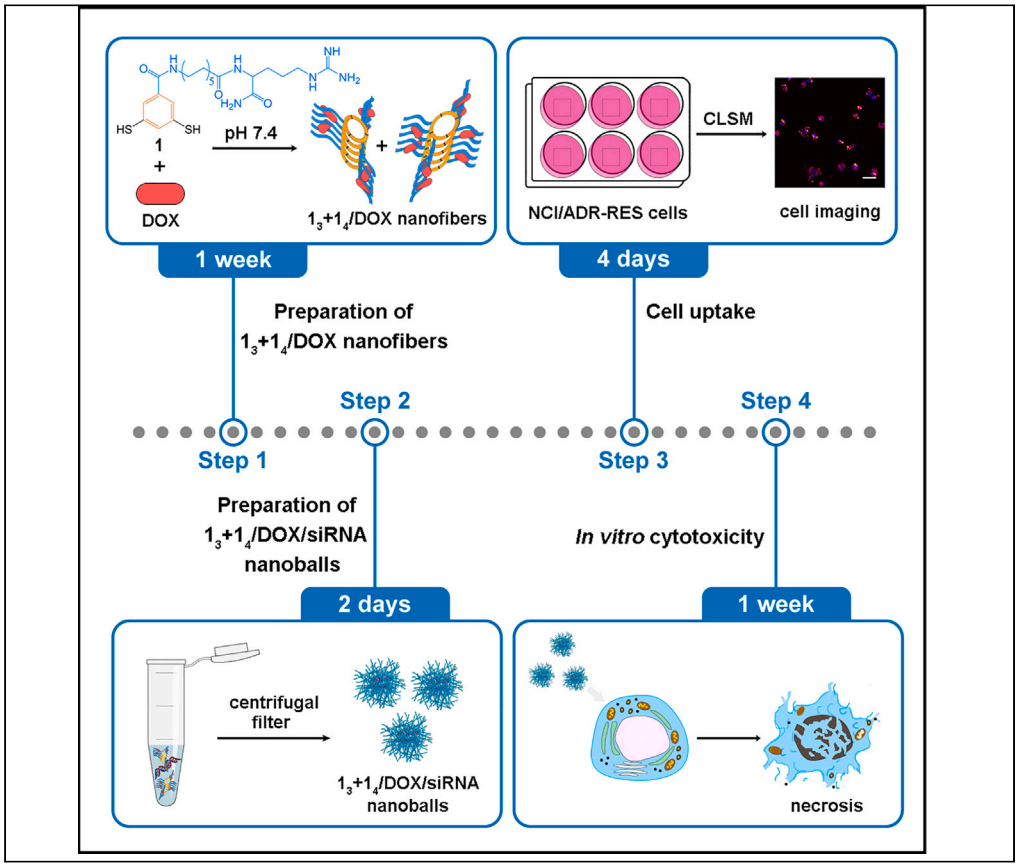
Protocol for preparing dynamic covalent macrocycles for co-delivering genes and drugs to cancer cell lines



Combination therapy using effective drug molecules and functional genes such as small interfering RNA (siRNA) has been suggested as a powerful strategy against multiple drug resistance. Here, we present a protocol for preparing a delivery system by developing dynamic covalent macrocycles using a dithiol monomer to co-deliver doxorubicin and siRNA. We describe steps for preparing the dithiol monomer, followed by co-delivery to form nanoparticles. We then detail procedures for cell uptake and assessing enhanced anti-cancer efficacy *in vitro*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Synthetic procedures of a dithiol monomer that can generate ring-like molecules

Prepare drug and gene co-delivery systems using the above macrocycles

Experimental details for cell uptake and *in vitro* anti-cancer efficacy

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Protocol for preparing dynamic covalent macrocycles for co-delivering genes and drugs to cancer cell lines

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SUMMARY

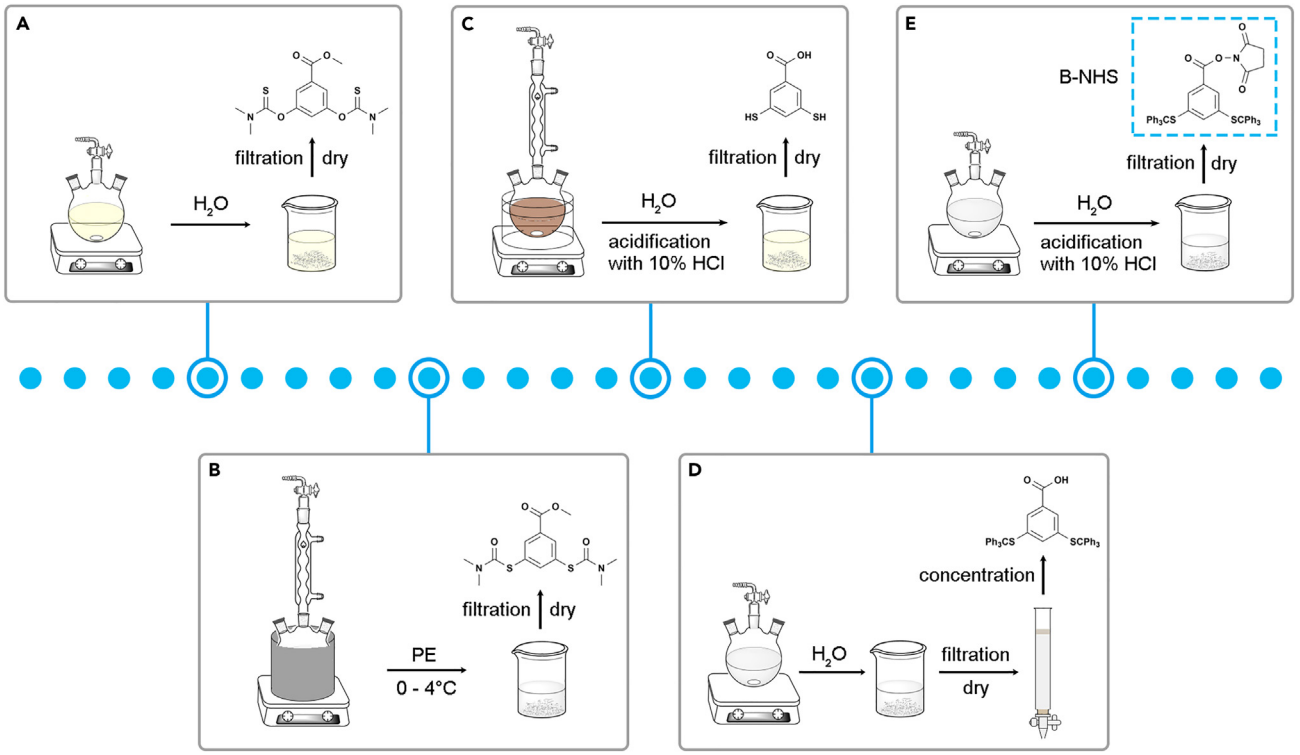
Combination therapy using effective drug molecules and functional genes such as small interfering RNA (siRNA) has been suggested as a powerful strategy against multiple drug resistance. Here, we present a protocol for preparing a delivery system by developing dynamic covalent macrocycles using a dithiol monomer to co-deliver doxorubicin and siRNA. We describe steps for preparing the dithiol monomer, followed by co-delivery to form nanoparticles. We then detail procedures for cell uptake and assessing enhanced anti-cancer efficacy *in vitro*. For complete details on the use and execution of this protocol, please refer to Lyu et al.[1]

BEFORE YOU BEGIN

At present, chemotherapy, a commonly used cancer treatment, is often associated with unsatisfactory clinical outcomes due to the phenomenon of multiple drug resistance (MDR). MDR is a phenomenon by which cancer cells have the ability to survive treatment with a variety of anti-cancer drugs.[2] There are many mechanisms responsible for MDR, adenosine triphosphate-binding (ATP-binding) cassette (ABC) transporters that can pump out chemotherapeutics are a typical kind of molecular mechanism.[3,4] Among ABC transporters, P-glycoprotein (P-gp), which is known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1), is overexpressed in many human cancer cells, such as breast, lung, and cervical cancer cells.[5,6] To overcome MDR at the genetic level, combination therapy with effective anti-cancer drugs and functional genes, such as small interfering RNA (siRNA), is a powerful strategy in cancer treatment.

To achieve the simultaneous delivery of siRNA and anti-cancer drugs, many functional delivery systems have been reported, including polymer-based nanoparticles,[7] liposomes,[8] and inorganic nanoparticles.[9] However, the main problems with polymeric delivery systems are their inefficient drug and gene release rates in cancer cells and low loading capacity.[10–12] Therefore, the design and synthesis of biocompatible and stable but responsive co-delivery systems with strong loading capacity are considered urgent.

Here, we developed a protocol for synthesizing dynamic covalent macrocycles to explore a gene and drug co-delivery system against drug-resistant cancer cells. The macrocycles (trimer and tetramer, $\mathbf{1}_3$+$\mathbf{1}_4$) are ring-like molecules by linking a dithiol monomer **1** through the thiol/disulfide exchange reaction. When associated with the drug molecule doxorubicin (DOX), $\mathbf{1}_3$+$\mathbf{1}_4$/DOX nanofibers are formed through non-covalent interactions. Through ionic interactions with siRNA, the nanofibers are subsequently kneaded into $\mathbf{1}_3$+$\mathbf{1}_4$/DOX/siRNA nanoballs. The drug and gene loading



Scheme 1. Schematic route of the start compound B-NHS

(A) General scheme of 3,5-bis(dimethylthiocarbamoyloxy)benzoic acid methyl ester.
(B) General scheme of 3,5-bis(dimethylcarbamoylsulfanyl)benzoic acid methyl ester.
(C) General scheme of 3,5-dimercaptobenzoic acid.
(D) General scheme of 3,5-bis(tritylthio)benzoic acid.
(E) General scheme of the starting compound B-NHS.

capability of 1_3+1_4/DOX/siRNA nanoballs is excellent, and as a result, the nanoballs exhibit enhanced cell uptake and anti-cancer ability *in vitro* compared with free DOX.

Preparation of the reagents

Timing: 2 weeks

The starting compound B-NHS can be synthesized via the following steps (Scheme 1) based on the previous literature,[1,13,14] and the details are followed.

1. Synthesis of 3,5-bis(dimethylthiocarbamoyloxy)benzoic acid methyl ester.
   a. Add methyl 3,5-dihydroxybenzoate (1.0 *g*, 5.94 mmol) and 1,4-diazabicyclo[2.2.2]octane (2.66 *g*, 23.8 mmol) into a 50 mL three-neck round bottom flask containing 8 mL of *N*,*N*-dimethylformamide (DMF).
   b. Vacuum the flask and back-fill with N_2. Repeat five times.

⚠ CRITICAL: Keeping the reaction in N_2 could avoid the production of by-products.

   c. Stir the mixture vigorously with a magnetic stirrer in an ice bath at 0°C for 30 min.
   d. Add a solution of *N*,*N*-dimethylthiocarbamoyl chloride (2.94 *g*, 23.8 mmol) in 8 mL of DMF dropwise with a disposable syringe into the flask at 0–4°C.

⚠ CRITICAL: If the mixture is too sticky to stir, dilute it with 5 mL of DMF.

e. Stir the reaction mixture with a magnetic stirrer vigorously at 25°C for 24 h.
f. Pour the mixture into 100 mL of deionized water in a beaker rapidly without stirring, then place the beaker in the fume hood. The white precipitate is formed in the beaker after 12 h.
g. Collect the final solid by vacuum filtration using a Buchner funnel with filter paper (5–13 μm pore size) and wash it with 96% ethanol (5 mL).
h. Obtain the white crystalline powder (yield 95%).
i. Analyze 3,5-bis(dimethylthiocarbamoyloxy)benzoic acid methyl ester by ^{1}H NMR (500 MHz, $CDCl_3$, 298K) δ (ppm): 7.64 (d, 2H), 7.06 (t, 1H), 3.90 (s, 3H), 3.45 (s, 6H), 3.35 (s, 6H).

2. Synthesis of 3,5-bis(dimethylcarbamoylsulfanyl)benzoic acid methyl ester.
a. Add 3,5-bis(dimethylthiocarbamoyloxy)benzoic acid methyl ester (1.0 *g*, 2.9 mmol) into a 50 mL three-neck round bottom flask containing 15 mL of diphenyl ether.
b. Vacuum the flask and back-fill with N_2. Repeat five times.
c. Stir the reaction mixture vigorously with a magnetic stirrer in a metal bath at 230–240°C for 3–4 h.

Note: The metal bath is used bismuth lead tin ingot (Rose's metal) as a heat transfer (melting point 94–98°C).

d. Analyze the reaction mixture by thin-layer chromatography (TLC) to confirm the completion of the reaction. (Dichloromethane (DCM)/Methanol (MeOH) = 9/1, Retention factors (Rf) = 0.4).
e. Remove the flask from the metal bath carefully and gradually cool it in the air to 30–40°C.
CAUTION: Hot! Take heat protection gloves when removing the flask from the metal bath.
f. Pour the reaction mixture into 50 mL of petroleum ether (PE) in a beaker rapidly without stirring.
g. Cover the beaker with plastic wrap to avoid the evaporation of PE.
h. Place the beaker in the 4°C cooling room/fridge. The light beige precipitate is formed in the beaker after 12 h.
i. Collect the light beige solid by filtration using a glass funnel with filter paper (5–13 μm pore size) and wash it with 10 mL of PE.
j. Obtain the light beige crystalline powder (yield 94%).
k. Analyze 3,5-bis(dimethylcarbamoylsulfanyl)benzoic acid methyl ester by ^{1}H NMR (500 MHz, $CDCl_3$, 298K) δ (ppm): 8.17 (d, 2H), 7.83 (t, 1H), 3.90 (s, 3H), 3.09 (br d, 6H), 3.03 (br d, 6H).

3. Synthesis of 3,5-dimercaptobenzoic acid troubleshooting 1.
a. Purge 1.75 M solution of KOH in 20 mL of diethylene glycol with N_2 for 2 h into a 50 mL three-neck round bottom flask.
b. Add 3,5-bis(dimethylcarbamoylsulfanyl)benzoic acid methyl ester (2.0 *g*, 5.85 mmol) into the flask.
c. Vacuum the flask and back-fill with N_2. Repeat five times.
d. Stir the reaction mixture vigorously with a magnetic stirrer in an oil bath at 105°C for 30 min.
e. After the reaction, cool the mixture to 25°C in the air.
f. Pour the reaction mixture into 100 mL of deionized water in a beaker rapidly and stir with a magnetic stirrer.
g. Rapidly acidify the solution to pH 3–4 by adding 15 mL of 10% hydrochloric acid (HCl) solution. The white precipitate is formed in the beaker.
h. Collect the final solid by vacuum filtration using a Buchner funnel with filter paper (5–13 μm pore size) and wash it with 20 mL of deionized water.
i. Obtain the white powder (yield 92%).
j. Analyze 3,5-dimercaptobenzoic acid by ^{1}H NMR (500 MHz, CD_3OD, 298K) *δ* (ppm): 7.38 (s, 1H), 7.64 (s, 2H).

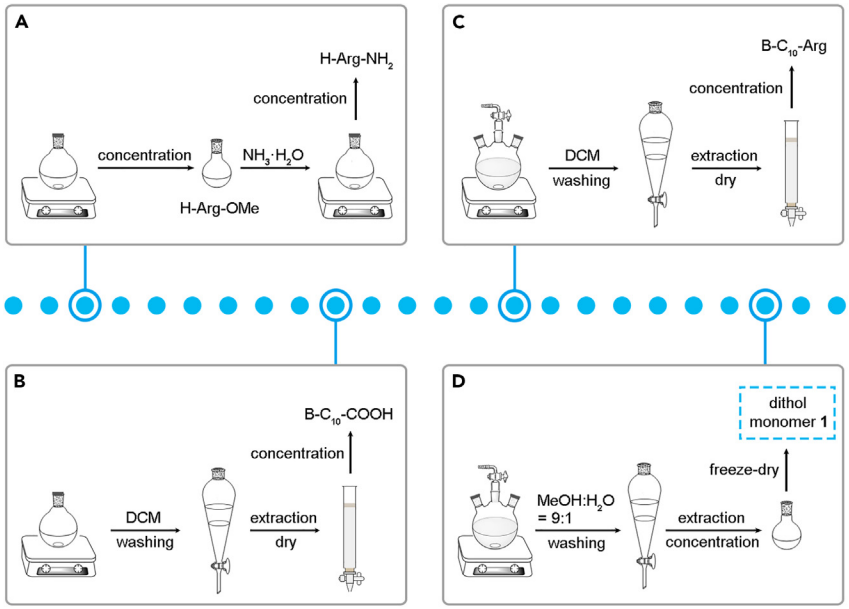
4. Synthesis of 3,5-bis(tritylthio)benzoic acid (B-COOH) troubleshooting 2.
   a. Add 3,5-dimercaptobenzoic acid (1.0 *g*, 5.37 mmol) and triphenylmethyl chloride (4.5 *g*, 16.2 mmol) into a 50 mL three-neck round bottom flask containing 15 mL of DMF.
   b. Vacuum the flask and back-fill with N_2. Repeat five times.
   c. Stir the reaction mixture with a magnetic stirrer vigorously at 25°C for 48 h.
   d. Analyze the reaction mixture by TLC to confirm the completion of the reaction (PE/Ethyl acetate (EA) = 1/1, Rf = 0.5).
   e. Pour the mixture into 40 mL of deionized water in a beaker rapidly and stir with a magnetic stirrer.
   f. Acidify the solution to pH 3–4 by adding 2 mL of 10% HCl solution, and the white precipitate is formed in the beaker.
   g. Collect the solid by vacuum filtration using a Buchner funnel with filter paper (5–13 μm pore size).
   h. Purify the crude product by pure chromatography system (flash column silica-12 *g*, chloroform).
   i. Collect the target product and concentrate by rotatory evaporation (40°C, 200 mbar, ~30 min) to afford the white solid (yield 91%).
   j. Analyze B-COOH by ^{1}H NMR (500 MHz, $CDCl_3$, 298K) δ (ppm): 7.34–7.32 (m, 12H), 7.29 (d, 2H), 7.24–7.18 (m, 18H), 7.04 (t, 1H).
5. Synthesis of B-NHS.
   a. Add B-COOH (1.610 *g*, 2.4 mmol) and *N*-hydroxysuccinimide (NHS) (1.104 *g*, 9.6 mmol) into a 250 mL three-neck round bottom flask containing 80 mL of DMF.
   b. Stir the mixture vigorously in an ice bath with a magnetic stirrer at 0°C for 30 min.
   c. Rapidly add *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) (1.84 *g*, 9.6 mol) to the flask.
   d. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 12 h.
   e. Analyze the reaction mixture by TLC to confirm the completion of the reaction (PE/EA = 5/1, Rf = 0.8).
   f. Rapidly pour the mixture into 200 mL of deionized water in a beaker and stir with a magnetic stirrer.
   g. Acidify the solution to pH 3–4 by adding 4 mL of 10% HCl solution, and the white precipitate is formed in the beaker.
   h. Collect the solid by vacuum filtration using a Buchner funnel with filter paper (5–13 μm pore size).
   i. Obtain the white solid (yield 86%).
   j. Analyze B-NHS by ^{1}H NMR (500 MHz, DMSO-d_6, 298K) δ (ppm): 7.26-7.20 (m, 30H), 7.09 (s, 1H), 7.06 (d, J = 1.50 Hz, 2H), 2.83 (s, 4H).

Synthesis of dithiol monomer 1

Timing: 2 weeks

Dithiol monomer **1** can be synthesized via the following steps (Scheme 2), and the details are followed. H-Arg-NH_2 is synthesized according to the previous literature.[15]

6. Synthesis of H-Arg-NH_2.
   a. Add *L*-arginine (2.613 *g*, 15 mmol) into a 50 mL round bottom flask containing 20 mL of MeOH.
   b. Stir the mixture vigorously in an ice bath with a magnetic stirrer at 0°C for 30 min.
   c. Add thionyl chloride ($SOCl_2$) (1.5 mL, 20 mmol) dropwise with a disposable dropping pipette into the flask.
   d. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 2 h.
   e. Remove the solvent by rotatory evaporation (30°C, 200 mbar, ~15 min) to afford H-Arg-OMe without purify.



Scheme 2. Schematic route of dithiol monomer 1

(A) General scheme of H-Arg-NH_2.

(B) General scheme of B-C_{10}-COOH.

(C) General scheme of B-C_{10}-Arg.

(D) General scheme of dithiol monomer **1**.

f. Add saturated ammonium hydroxide aqueous solution (10 mL, 65 mmol) into the flask rapidly.

g. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 12 h.

h. Concentrate the solution by rotatory evaporation (50°C, 100 mbar, ~20 min) to afford the crude product.

i. Purify the product by washing it with 20 mL of ice ethyl ether.

j. Obtain the white solid (yield 96%).

k. Analyze H-Arg-NH_2 by ^{1}H NMR (500 MHz, D_2O, 298K) δ (ppm): 3.98 (t, 1H), 3.28 (t, 2H), 1.96-1.90 (m, 2H), 1.75-1.69 (m, 2H).

7. Synthesis of B-C_{10}-COOH.

a. Add B-NHS (650 mg, 0.84 mmol), 11-aminoundecanoic acid (844 mg, 4.2 mmol), and triethylamine (TEA) (588 μL, 4.2 mmol) successively into a 50 mL round bottom flask containing 20 mL of DMF.

b. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 24 h.

c. Analyze the reaction mixture by TLC to confirm the completion of the reaction (DCM/MeOH = 15/1, Rf = 0.2).

d. Pour the mixture into 50 mL of DCM in a beaker rapidly.

e. Transfer the solution to a separating funnel, then wash with 10% HCl (30 mL), saturated sodium hydrogen carbonate ($NaHCO_3$) solution (30 mL), and deionized water (30 mL) to remove TEA and DMF.

f. Transfer the organic layer to a 100 mL conical flask and add anhydrous magnesium sulfate ($MgSO_4$) (1 *g*) in the flask.

g. Stir the mixture slowly with a magnetic stirrer for 2 h.

h. Filter the solution by a glass funnel with filter paper (5–13 μm pore size) in a 50 mL round bottom flask.

i. Remove the solvent by rotatory evaporation (30°C, 200 mbar, ~20 min) to afford the dried crude product.

j. Purify the crude product by pure chromatography system (flash column silica-12 *g*, DCM/MeOH gradient from 30/1 to 5/1).

k. Collect the target product and concentrate by rotatory evaporation (30°C, 200 mbar, ~30 min) to afford the white solid (yield 78%).
l. Analyze B-C_{10}-COOH by ^{1}H NMR (500 MHz, $CDCl_3$, 298K) δ (ppm): 7.32-7.29 (m, 12H), 7.19-7.17 (m, 18H), 7.09 (t, 1H), 6.80 (d, 2H), 5.11 (t, 1H), 3.20-3.19 (q, 2H), 2.35 (t, 2H), 1.64 (p, 2H), 1.45 (p, 2H), 1.37-1.29 (m, 12 H). ^{13}C NMR (125 MHz, $CDCl_3$, 298K) δ (ppm): 177.36, 166.06, 144.30, 142.23, 135.02, 134.35, 132.26, 130.08, 127.92, 126.92, 71.38, 39.95, 33.78, 29.65, 29.47, 29.38, 29.27, 29.20, 29.08, 26.00, 24.83. ESI-QTOF $[M+Na]^+$ found: 876.3585 (expected:876.3521).

8. Synthesis of B-C_{10}-Arg troubleshooting 3.
a. Add B-C_{10}-COOH (717 mg, 0.84 mmol) and H-Arg-NH_2 (295 mg, 1.7 mmol) into a 50 mL three-neck round bottom flask containing 15 mL of DMF.
b. Stir the mixture vigorously in an ice bath with a magnetic stirrer at 4°C for 30 min.
c. Add 1-hydroxybenzotriazole hydrate (229.5 mg, 1.7 mmol), EDC·HCl (326.4mg, 1.7 mmol), and *N,N*-diisopropylethylamine (DIPEA) (457 μL, 3.4 mmol) into the flask successively.
d. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 24 h.
e. Analyze the reaction mixture by TLC to confirm the completion of the reaction (DCM/MeOH = 4/1, Rf = 0.4).
f. Pour the mixture into 50 mL DCM in a beaker rapidly.
g. Transfer the solution to a separating funnel, then wash with 10% HCl (30 mL), saturated $NaHCO_3$ solution (30 mL), and deionized water (30 mL) to remove DIPEA and DMF.
h. Transfer the organic layer to a 100 mL conical flask and add anhydrous $MgSO_4$ (1 *g*) in the flask.
i. Stir the mixture slowly with a magnetic stirrer for 2 h.
j. Filter the solution by a glass funnel with filter paper (5–13 μm pore size) in a 50 mL round bottom flask.
k. Remove the solvent by rotatory evaporation (30°C, 200 mbar, ~20 min) to afford the dried crude product.
l. Purify the crude product by pure chromatography system (flash column silica-12 *g*, DCM/MeOH gradient from 10/1 to 1/1).
m. Collect the target product and concentrate by rotatory evaporation (30°C, 200 mbar, ~15 min) to afford the white solid (yield 58%).
n. Analyze B-C_{10}-Arg by ^{1}H NMR (500 MHz, $CDCl_3$, 298K) δ (ppm): 7.32-7.28 (m, 12H), 7.20-7.13 (m, 19H), 6.78 (s, 2H), 5.39 (br s, 1H), 3.28 (br s, 2H), 3.12 (br s, 2H), 2.24 (br s, 2H), 1.92-1.70 (m, 4H), 1.51 (br s, 2H), 1.39 (br s, 2H), 1.25-1.19 (m, 12H). ^{13}C NMR (125 MHz, $CDCl_3$, 298K) δ (ppm):147.00, 145.40, 144.24, 135.06, 132.46, 130.04, 129.82, 128.08, 127.92, 127.86, 127.41, 126.93, 71.43, 53.84, 40.19, 37.29, 35.73, 29.74, 29.55, 29.47, 27.11, 18.83. ESI-QTOF $[M+H]^+$ found: 1009.4840 (expected:1009.4873).

9. Synthesis of dithiol monomer **1** troubleshooting 4.
a. Add B-C_{10}-Arg (100 mg, 99.1 mmol) into a 25 mL three-neck round bottom flask.
b. Purge N_2 into the flask for 2 h.
c. Add 3 mL of trifluoroacetic acid (TFA) with a disposable syringe into the flask rapidly.
d. Sonicate the flask for 5 min.
e. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 15 min.
f. Add triethylsilane (200 μL, 1.25 mmol) with a disposable syringe into the flask rapidly.
g. Stir the reaction mixture vigorously with a magnetic stirrer at 25°C for 15 min.

Note: The reaction mixture changes from the orange solution to colorless with the white precipitate.

h. Remove TFA by purging N_2 into the flask.
i. Add 20 mL of a degassed mixture of MeOH/H_2O = 9/1 in the flask.

⚠ CRITICAL: The mixture of MeOH/H_2O = 9/1 should be degassed and backfilled with N_2 three times before use.

j. Transfer the solution to a separating funnel, and wash with PE (3 × 10 mL).
k. Transfer the aqueous layer to a 50 mL round bottom flask, then remove MeOH by rotatory evaporation (30°C, 200 mbar, ~15 min).
l. Freeze-dried the solution for 48 h to afford the white pure product (yield 59%).
m. Analyze dithiol monomer **1** by ^{1}H NMR (500 MHz, CD_3OD, 298K) δ (ppm): 7.38 (s, 2H), 7.31 (s, 1H), 4.33 (q, 1H), 3.16-3.15 (q, 2H), 2.21 (t, 2H), 1.81 (m, 1H), 1.71-1.63 (m, 6H), 1.32-1.28 (m, 12 H). ^{13}C NMR (125 MHz, CD_3OD, 298K) δ (ppm): 176.55, 176.41, 168.94, 158.64, 137.44, 135.29, 131.76, 124.99, 53.58, 41.97, 41.04, 36.88, 30.55, 30.50, 30.39, 30.34, 30.31, 28.01, 26.86, 26.36. ESI-QTOF $[M+H]^+$ found: 525.2641 (expected: 525.2682).

Preparation of culture cells

Timing: 2 weeks

10. Prepare the complete cell culture medium.

Note: Refer to materials and equipment. Conduct cell uptake and *in vitro* cytotoxicity in the complete cell culture medium.

11. Prepare NCI/ADR-RES complete medium.

Note: Refer to materials and equipment. Resuscitate and culture NCI/ADR-RES cells in NCI/ADR-RES complete medium.

12. Resuscitate and culture DOX-resistant NCI/ADR-RES cell line troubleshooting 5.
    a. Pre-warm trypsin-EDTA solution, PBS, and NCI/ADR-RES complete medium to 37°C.
    b. Preserve the cryopreservation vial containing NCI/ADR-RES cell line in a liquid nitrogen transport vessel to maintain the vial at a low temperature before resuscitating the cells.
    c. Transfer the vial to a 37°C water bath quickly for 1–2 min until no ice crystals remain.

⚠ CRITICAL: Rapidly thawing is essential to minimize damage to the cells. Do not immerse the vial in the water bath totally, because it may cause contamination of the cells.

    d. Wipe the entire vial with a tissue soaked in 70% ethanol before opening it.
    e. Pipette the whole cell suspension of the vial into a 15 mL centrifuge tube and add 10 mL of PBS to it.
    f. Centrifuge (300 × *g*, 5 min, 25°C) and remove the supernatant.
    g. Gently resuspend the cell pellet in 5 mL of NCI/ADR-RES complete medium.
    h. Transfer the cell suspension to a T25 cell culture flask.
    i. Place the T25 cell culture flask in an incubator with 5% CO_2 at 37°C.

⚠ CRITICAL: After culturing the cells in the incubator for 24 h, observe the cells under the microscope. There may be some dead cells floating in the cell flask, because of thawing and centrifuging. If it is, remove and discard the culture medium from the cell flask. After supplementing with 5 mL of the NCI/ADR-RES complete medium, place the cell flask in the incubator with 5% CO_2 at 37°C.

    j. Observe the cells under the microscope. When the cells reach 80–90% confluence, remove the cell culture medium from the T25 cell flask.
    k. Gently rinse the cells with 2 mL of PBS and remove the supernatant.

l. Add 1 mL of trypsin-EDTA to the cell flask.
m. Gently swirl the content to cover the cell layer, then remove the trypsin-EDTA.
n. Incubating the cell flask in the incubator at 37°C for 3–4 min.
o. Observe the cells under the microscope. The detached cells appear rounded and refractile.

Note: NCI/ADR-RES cells are firmly adherent in the cell flask, and can be detached quickly at 37°C.

⚠ CRITICAL: If less than 90% of cells are detached, incubate the flask at 22–25°C for another 1 min.

p. Once the cells are detached, add 5 mL of NCI/ADR-RES complete medium into the flask.
q. Gently disperse the medium by pipetting over the cell layer surface several times to ensure the recovery of cells.
r. Remove and discard 3 mL of the cell suspension.
s. Add 3 mL of NCI/ADR-RES complete medium in the cell flask.
t. Gently disperse the cell suspension by pipetting several times.
u. Place the cell flask in the incubator with 5% CO_2 at 37°C.
v. Continue to sub-culture cells, maintaining 80–90% confluence, until ready for construction of stable cell lines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
N,N-dimethylformamide, 99%	VWR	Cat#23466.298
Ethanol, 96%	VWR	Cat#1.59010
Ethanol, 70%	VWR	Cat#85825.360
Diphenyl ether, 99%	TCI	Cat#P0177
Dichloromethane, 99.5%	Fisher Chemical	Cat#11458173
Methanol, 99.9%	Sigma-Aldrich	Cat#34860
Petroleum ether 40°C–60°C, 99%	Fisher Chemical	Cat#11433633
Ethyl acetate, 99.5%	Sigma-Aldrich	Cat#34858
Chloroform, 99%	VWR	Cat#22711.324
Hydrochloric acid, 37%	Fisher Chemical	Cat#10724641
Acetonitrile, LC-MS grade	Fisher Chemical	Cat#10001334
Methyl 3,5-dihydroxybenzoate	Acros	Cat#170440250
1,4-Diazabicyclo[2.2.2]octane (DABCO)	TCI	Cat#D0134
N,N-dimethylthiocarbamoyl chloride, 97%	Sigma-Aldrich	Cat#135895
Potassium hydroxide (KOH)	Fisher Scientific	Cat#11413693
Sodium hydroxide (NaOH)	Sigma-Aldrich	Cat#1.06498
Diethylene glycol, 99.5%	TCI	Cat#D0495
Triphenylmethyl chloride, 98%	TCI	Cat#C0308
N-hydroxysuccinimide (NHS), 98%	TCI	Cat#H0623
N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl), 98%	Abcr	Cat#AB181824
L-arginine, 99%	Fisher Scientific	Cat#11498850
Thionyl chloride ($SOCl_2$), 97%	Sigma-Aldrich	Cat#320536
Ammonium hydroxide aqueous solution, 30–33%	Fisher Scientific	Cat#15690770

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
11-Aminoundecanoic acid, 97%	Sigma-Aldrich	Cat#A82605
Triethylamine (TEA), 99%	Sigma-Aldrich	Cat#T0886
1-Hydroxybenzotriazole hydrate (HOBt), 97%	Sigma-Aldrich	Cat#54802
N,*N*-diisopropylethylamine (DIPEA), 99%	TCI	Cat#D1599
Trifluoroacetic acid (TFA), 99%	Fisher Scientific	Cat#11404863
Deuterated chloroform ($CDCl_3$)	Eurisotop	Cat#D006H
Deuterated oxide (D_2O)	Eurisotop	Cat#D214F
Deuterated dimethyl sulfoxide (DMSO-d_6)	Eurisotop	Cat#D010F
Deuterated methanol (CD_3OD)	Eurisotop	Cat#D024F
Formic acid (FA), 99%	Fisher Scientific	Cat#10596814
Triethylsilane, 98%	TCI	Cat#T0662
Doxorubicin (DOX)	Melone Pharmaceutical	CAS:25316-40-9
Paraformaldehyde, 90%	TCI	Cat#P0018
3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), 98%	TCI	Cat#D0801
Fetal bovine serum (FBS)	Fisher Scientific	Cat#15575309
L-glutamine	Fisher Scientific	Cat#15410314
Penicillin-streptomycin	Fisher Scientific	Cat#11568876
PBS	Fisher Scientific	Cat#11629980
Tryspin-EDTA	Fisher Scientific	Cat#11570626
Dulbecco's Modified Eagle Medium (DMEM)	Fisher Scientific	Cat#11625200
Hoechst	Fisher Scientific	Cat#11534886
Mounting medium	Fisher Scientific	Cat#15820100
Critical commercial assays		
Cell apoptosis kit with Annexin V	Fisher Scientific	Cat#10257392
Experimental models: Cell lines		
Human: NCI/ADR-RES cell line	Universitat de Girona, and Biomedical Research Institute of Girona	N/A
Oligonucleotides		
P-gp siRNA 5′-GCA CUA AAG UAG GAG ACA AdTdT-3′	GenePharma	N/A
FAM-siRNA 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′	GenePharma	N/A
Software and algorithms		
ChemDraw Professional 19.1	PerkinElmer	https://www.perkinelmer.com/category/chemdraw
TopSpin 4.1.4	Bruker	https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html
ImageJ	National Institutes of Health	https://imagej.net/ij/index.html
Flowjo V10	BD	https://www.flowjo.com/solutions/flowjo/downloads
Other		
Analytical balance	AB135-S/FACT	Mettler Toledo
Magnetic stir plate	MR Hei-Standard Hei-PLATE sensor	Heidolph
Rotary evaporator	Hei-VAP Ultimate	Heidolph
pH meter	Mettler Toledo	FiveEasy F20
Pure chromatography system	Buchi	C-850 FlashPrep
Freezer-dryer	ZIRBUS	VaCo 2
Nuclear magnetic resonance spectroscopy (NMR)	Bruker	500 MHz

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mass spectrometry (ESI-TOF)	Bruker	micrOTOF-Q ESI
High performance liquid chromatography (HPLC)	Agilent	Agilent 1100
Ultraviolet-Vis (UV-Vis) absorption spectroscopy	PerkinElmer Lambda	N/A
Transmission electron microscopy (TEM)	JEM 1400 plus	N/A
Eppendorf centrifuge	Eppendorf	5810R
Automated cell counter	Bio-Rad	TC20
Flow cytometer	BD	LSRFortessa
Confocal laser scanning microscope	Zeiss	LSM880
Cell imaging reading	Agilent	Cytation 5
Copper grid	Sigma-Aldrich	TEM-CF200CU50
1 mL syringe	Fisher Scientific	Cat#15489199
Filter paper (5–13 μm pore size)	Fisher Scientific	Cat#11714166
TLC silica gel 60 F254	Sigma-Aldrich	Cat#1.05554.0001
Flash column silica-CS (12 *g*)	Agela	Cat#CS140012-0
Dialysis bag	Tansoole	Cat#02037433
Reversed-phase column	Waters	Cat#WAT200630
Ultra-4 centrifugal filter unit	Sigma-Aldrich	Cat#UFC810008
T25 cell culture flask	Corning	Cat#430639
Glass square coverslips	Fisher Scientific	Cat#12323128
Glass slide	Fisher Scientific	Cat#11844782
Cell culture plate, 96 well	Corning	Cat#3596
Cell culture plate, 6 well	Corning	Cat#3506
Centrifuge tube (15 mL)	Fisher Scientific	Cat#11849650
Microcentrifuge tube (1.5 mL)	Fisher Scientific	Cat#11558232
5 mL round-based test tube	Fisher Scientific	Cat#11729412
10 mL pipette	Corning	Cat#4488

MATERIALS AND EQUIPMENT

The complete cell culture medium

Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	88.57%	500 mL
Fetal Bovine Serum (FBS)	8.86%	50 mL
Penicillin-Streptomycin	53.14 U/mL	3 mL
L-glutamine	4.07 mM	11.5 mL
Total	**N/A**	**564.5 mL**

Store at 4°C up to 3 months.

NCI/ADR-RES complete medium

Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	88.57%	500 mL
Fetal Bovine Serum (FBS)	8.86%	50 mL
Penicillin-Streptomycin	53.14 U/mL	3 mL
L-glutamine	4.07 mM	11.5 mL
Doxorubicin (DOX)	3 μM	0.92 mg
Total	**N/A**	**564.5 mL**

Store at 4°C up to 3 months.

STEP-BY-STEP METHOD DETAILS

Step 1: Preparation of 1_3+1_4/DOX nanofibers

Timing: 1 week

High performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), ultraviolet-vis (UV-Vis), and transmission electron microscopy (TEM) are employed to characterize 1_3+1_4/DOX nanofibers.

1. Preparation of 1_3+1_4/DOX nanofibers.
   a. Dissolve DOX (100 mg) in 1 mL deionized H_2O to prepare DOX solution.
   b. Dissolve monomer **1** (0.21 mg) in a 2 mL glass vial containing pH 7.4 PBS (1 mL).

   ⚠ CRITICAL: When dissolving monomer 1 in PBS, add 100 mM of NaOH solution (1–2 μL) to adjust pH to 7.4.

   c. Add DOX solution (2 μL) into the glass vial containing monomer **1**.
   d. Dissolve monomer **1** (0.105 mg) in another 2 mL glass vial containing pH 7.4 PBS (0.5 mL).

   Note: The monomer **1** solution is the control group for HPLC and LC-MS analysis.

   e. Wrap the vials with aluminum foil wrap.
   f. Stir the vials slowly with a magnetic stirrer at 25°C for 3 days.
   g. Analyze the mixture by HPLC to confirm the complete oxidation of **1**.
      i. Dilute 10 μL of the mixture to a 1:20 dilution in 5% dimethyl sulfoxide (DMSO) containing 5% of TFA.
      ii. Analyze the sample by HPLC using a reversed-phase HPLC column (Waters, C8 Column) in Figure 1A. UV absorbance is monitored at 254 nm, the column temperature is kept at 25°C, and the eluent flow is 1.0 mL/min. The method is shown in Table 1.
   h. The HPLC results of the mixture are shown in Figure 1B.
   i. Use LC-MS to characterize the components of the mixture (Figures 1C and 1D). The LC method is the shown in Table 1.
   j. After complete oxidation, transfer the mixture into the dialysis bag (3500 Da), and dialyze for 1 day.
   k. Freeze-dried the solution for 48 h to afford 1_3+1_4/DOX.
   l. Use UV-vis to characterize the DOX content at 480 nm. Calculate the drug-loading content (DLC) according to the following equation:

$$DLC(\%) = \frac{W_{DOX}}{W_{DOX}+W_{1_3+1_4}} \times 100$$

Where W_{DOX} and $W_{1_3+1_4}$ represent the weight of encapsulated DOX and the weight of the macrocycles, respectively.

2. Prepare the samples of 1_3+1_4/DOX nanofibers for TEM observation.
   a. Prepare a new 200 mesh carbon-coated copper grid for TEM.
   b. Prepare 1_3+1_4/DOX solution in 1 mL of deionized H_2O (0.05 mg/mL).
   c. Drop 5 μL of 1_3+1_4/DOX solution on the copper grid.
   d. Remove the excess sample solution on the copper grid with a piece of filter paper after 5 min. Repeat twice.
   e. Dry the copper grid at 25°C for 3 h.
   f. Observe the sample by TEM with the voltage of 80.0 kV (Figure 2A).

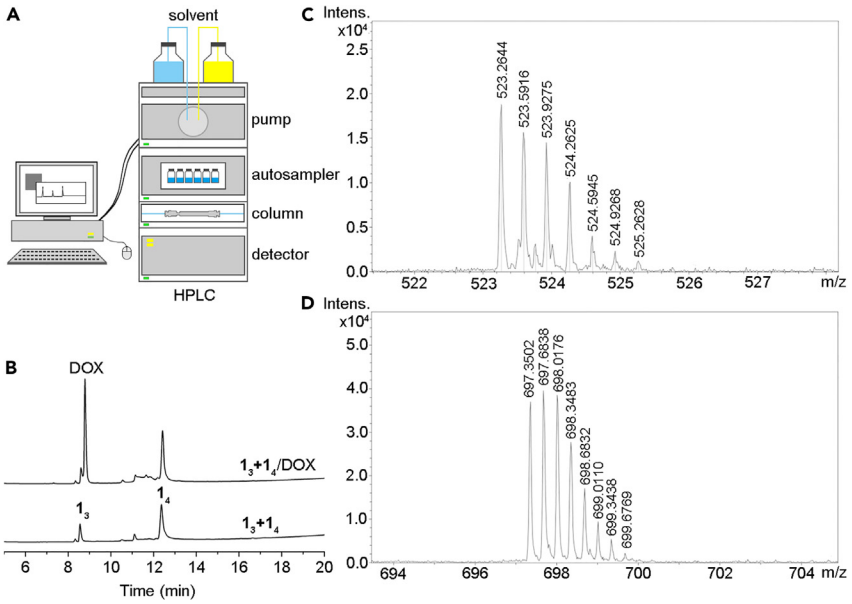


Figure 1. Characterization of 1_3+1_4 and 1_3+1_4/DOX by HPLC and LC-MS

(A) HPLC instrument for analysis.

(B) HPLC result of 1_3+1_4 and 1_3+1_4/DOX in PBS buffer (pH 7.4).

(C) Mass spectrum of **trimer** 1_3 m/z calculated for $[M+3H]^{3+}$, 523.2525, found 523.2644.

(D) Mass spectrum of **tetramer** 1_4 m/z calculated for $[M+3H]^{3+}$, 697.6686, found 697.6838. Figures 1B–1D reprinted with permission from Lyu et al.[1]

Step 2: Preparation of 1_3+1_4/DOX/siRNA nanoballs

Timing: 2 days

TEM is employed to characterize 1_3+1_4/DOX/siRNA nanoballs.

3. Preparation of 1_3+1_4/DOX/siRNA nanoballs.
   a. Dissolve 2 mg of 1_3+1_4/DOX in 1 mL of deionized H_2O and sonicate for 10 min.
   b. Incubate 1_3+1_4/DOX solution with siRNA solution for 1 h to form the 1_3+1_4/DOX/siRNA assembly (N/P = 6, siRNA = 50 nM). The N/P ratio represents the molar ratio of nitrogen in 1_3+1_4/DOX cationic to phosphate in siRNA.
   c. Purify 1_3+1_4/DOX/siRNA assembly by ultra-4 centrifugal filter devices and centrifuge (400 × *g*, 10 min, 25°C).
   d. Disperse 1_3+1_4/DOX/siRNA assembly in 1 mL of deionized H_2O for the following experiments.
4. Prepare the samples of 1_3+1_4/DOX/siRNA nanoballs for TEM observation.
   a. Prepare a new 200 mesh carbon-coated copper grid for TEM.
   b. Prepare 1_3+1_4/DOX/siRNA solution into 1 mL of deionized H_2O (0.05 mg/mL).
   c. Drop 5 μL of 1_3+1_4/DOX/siRNA solution on the copper grid.

Table 1. Gradient elution method in HPLC for analyzing the sample

Time (min)	A: Acetonitrile (0.1 v% Formic acid)	A: Water (0.1 v% Formic acid)
0	5	95
20	95	5
25	95	5
30	5	95

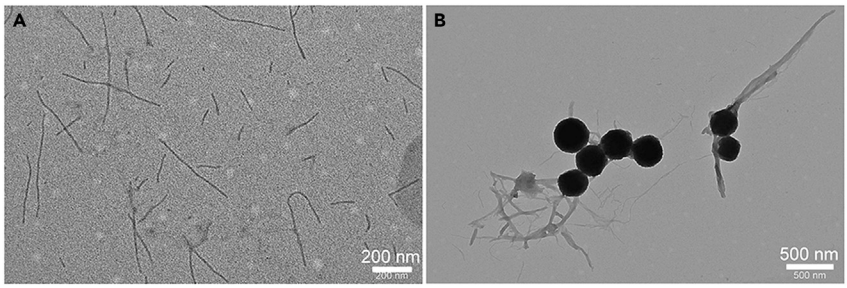


Figure 2. TEM analysis of 1_3+1_4/DOX and 1_3+1_4/DOX/siRNA

(A) 1_3+1_4/DOX nanofibers; Scale bar, 200 nm.

(B) 1_3+1_4/DOX/siRNA nanoballs; Scale bar, 500 nm. Figure reprinted with permission from Lyu et al.[1]

d. Remove the excess sample solution on the copper grid with a piece of filter paper after 5 min. Repeat twice.
e. Dry the copper grid at 25°C for 3 h.
f. Observe the sample by TEM with the voltage of 80.0 kV (Figure 2B).

Step 3: Cell uptake

Timing: 4 days

Confocal laser scanning microscopy (CLSM) is employed to investigate the cellular uptake mediated with 1_3+1_4/DOX/siRNA co-delivery system.

5. Prepare the 6-well dish with coverslips for cell imaging.
   a. Place some square coverslips (18 × 18 mm) in a beaker with 30 mL of ddH_2O, and sonicate the beaker for 15 min.
   b. Remove and discard ddH_2O.
   c. Add 30 mL of 70% ethanol in the beaker, and cover the beaker with plastic wrap.
   d. Add 50 μL of ddH_2O in the center of each well of a 6-well dish.
   e. Take a prepared coverslip from the beaker with tweezers carefully and burn it with a blast burner quickly to evaporate ethanol.

   Alternatives: Drying the coverslip in a UV-irradiated bio-clean bench is also a useful method.

   f. After the coverslip cools down, place it in the center of well in the 6-well dish carefully.

   Note: The coverslip could attach the dish tightly due to the surface tension of water.

6. Detach NCI/ADR-RES cells.
   a. Take out the T25 cell flask (culture in step [preparation of culture cells]) where cells are in the 80–90% confluence (about 10^6 cells/T25 flask) from the incubator and put it into the bio-clean bench.
   b. Remove and discard the culture medium from the flask.
   c. Gently rinse the cells with 2 mL of PBS and remove the supernatant.
   d. Add 1 mL of trypsin-EDTA to the cell flask.
   e. Gently swirl the content to cover the cell layer, then remove the trypsin-EDTA.
   f. Incubate the cell flask in the incubator at 37°C for 3–4 min.
   g. Observe the cells under the microscope. The detached cells appear rounded and refractile.
   h. Once the cells detached, add 5 mL of the complete culture medium in the cell flask.

i. Gently disperse the medium by pipetting over the cell layer surface several times to ensure the recovery of the cells.
j. Mix 10 μL of cell suspension and 10 μL of 0.4% of trypan blue solution in a 1.5 mL microcentrifuge tube.
k. Incubate the mixture at 37°C for 3–4 min.
l. Apply a drop of the trypan blue/cell mixture to a counting slide and count the cells with a TC20 automated cell counter.

7. Co-culture NCI/ADR-RES cells with free DOX, FAM-siRNA, 1_3+1_4/DOX, and 1_3+1_4/DOX/FAM-siRNA troubleshooting 6.
   a. Seed NCI/ADR-RES cells into the prepared 6-well dish with coverslips (5×10^4 cells/well) with the complete cell culture medium.
   b. Culture the cells in the incubator at 37°C for 12 h.
   c. Prepare 2 mL of free DOX (2 μg/mL), FAM-siRNA (50 nM), 1_3+1_4/DOX (DOX 2 μg/mL), and 1_3+1_4/DOX/FAM-siRNA (DOX 2 μg/mL, FAM-siRNA 50 nM) diluted in the complete cell culture medium separately.
   d. Take out the 6-well dish from the incubator and put it into the bio-clean bench.
   e. Remove and discard the culture medium from the 6-well dish.
   f. Add 2 mL of free DOX, FAM-siRNA, 1_3+1_4/DOX, 1_3+1_4/DOX/FAM-siRNA solutions, and the complete cell culture medium into the well of the 6-well dish separately.

   Note: The cells in the complete cell culture medium are the control group.

   g. Incubate the 6-well dish in the incubator at 37°C for 4 h.
8. Observe NCI/ADR-RES cells by CLSM (Figure 3A).
   a. Take out the 6-well dish from the incubator and put it into the bio-clean bench.
   b. Remove the supernatant in the 6-well dish.
   c. Wash the cells in each well with 1 mL of PBS 3 times separately.
   d. Fix the cells with 1 mL of 4% v/v paraformaldehyde for 10 min.
   e. Wash the cells with 1 mL of PBS three times to remove paraformaldehyde.
   f. Stain the nucleus of the cells with 200 μL of Hoechst (10 μg/mL) for 10 min.
   g. Wash the cells with 1 mL of PBS three times to remove Hoechst.
   h. Take glass microscope slides and wipe them with 70% ethanol for use.
   i. Add 20 μL of mounting medium on the center of the glass slide.
   j. Take out the coverslip from the 6-well dish with tweezers carefully and dip it in a beaker containing 20 mL of ddH_2O for 1 s to remove PBS.
   k. Put the coverslip upside-down and bring it down on the glass slide until it touches the mounting medium.
   l. Place the glass slides with the cells in the fume hood to dry for 1 h.
   m. Seal the edges of the coverslip with clear nail polish and wait to dry.
   n. Observe the samples under CLSM.

Step 4: *In vitro* cytotoxicity

Timing: 1 week

MTT assay and Cell apoptosis kit with Annexin V are employed to determine the synergistic efficacy of 1_3+1_4/DOX/siRNA against NCI/ADR-RES cells *in vitro*.

9. Co-culture NCI/ADR-RES cells with free DOX, 1_3+1_4/DOX, and 1_3+1_4/DOX/P-gp siRNA.
   a. Perform the cell culture as described in step [6. Detach NCI/ADR-RES cells].
   b. Seed NCI/ADR-RES cells into 96-well dishes (8×10^3 cells/well) with the complete cell culture medium.
   c. Culture the cells in the incubator at 37°C for 12 h.

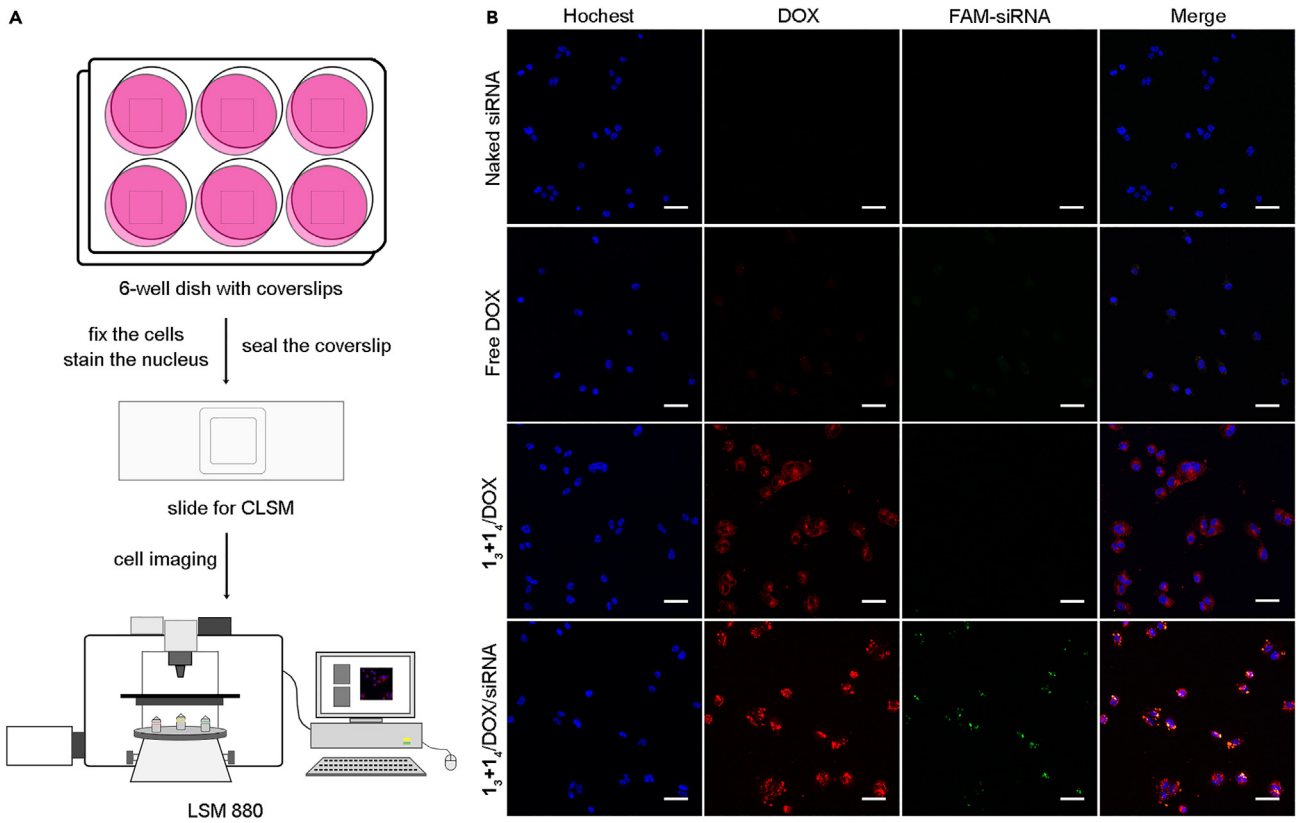


Figure 3. Cell uptake experiment

(A) Prepare cell sample for CLSM.

(B) Fluorescence microscopy images of NCI/ADR-RES cells incubated with different formulations. Green, red, and blue fluorescence indicated FAM-siRNA, DOX, and the nucleus, respectively; Scale bar, 50 μm. Figure 3B reprinted with permission from Lyu et al.[1]

d. Prepare free DOX (10–100 μg/mL), $\mathbf{1_3}$+$\mathbf{1_4}$/DOX (DOX 10–100 μg/mL), and $\mathbf{1_3}$+$\mathbf{1_4}$/DOX/P-gp siRNA (DOX 10–100 μg/mL, P-gp siRNA 50 nM) diluted in the complete cell culture medium separately.
e. Take out the 96-well dishes from the incubator and put them into the bio-clean bench.
f. Remove the culture medium from the 96-well dishes.
g. Add 100 μL of free DOX, $\mathbf{1_3}$+$\mathbf{1_4}$/DOX, $\mathbf{1_3}$+$\mathbf{1_4}$/DOX/P-gp siRNA, and the complete cell culture medium into the well of the 96-well dishes separately.
h. Incubate the 6-well dish in the incubator with 5% CO_2 at 37°C for 48 h.

10. Measure and calculate cell viability.
a. Take out the 96-well dishes from the incubation.
b. Add 10 μL of MTT solution (5 mg/mL) into each well of the 96-well dishes.
c. After 3 h incubation, replace the culture medium with 100 μL of DMSO in each well of the 96-well dishes.
d. Analyze the 96-well dish by the cell imaging reader. The optical density (OD) with the excitation wavelength of 570 nm. The cell viability (CV) is calculated by the equation as followed:

$$CV(\%) = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}}$$

Where OD_{blank}, OD_{sample} and $OD_{control}$ represent the OD values of blank group, treatment group and control group, respectively.

11. Apoptosis Analysis.

a. Perform the cell culture as described in step [6. Detach NCI/ADR-RES cells].
b. Seed NCI/ADR-RES cells into a 6-well dish (10^5 cells/well) with the complete cell culture medium.
c. Culture the cells in the incubator at 37°C for 12 h.
d. Prepare free DOX (50 μg/mL), P-gp siRNA (50 nM), $\mathbf{1}_3$+$\mathbf{1}_4$ (0.1 mM), $\mathbf{1}_3$+$\mathbf{1}_4$/DOX (DOX 50 μg/mL) and $\mathbf{1}_3$+$\mathbf{1}_4$/DOX/P-gp siRNA (DOX 50 μg/mL, FAM-siRNA 50 nM) diluted in the complete cell culture medium separately.
e. Take out the 6-well dish from the incubator and put it into the bio-clean bench.
f. Remove and discard the culture medium from the 6-well dish.
g. Add 2 mL of free DOX, P-gp siRNA, $\mathbf{1}_3$+$\mathbf{1}_4$, $\mathbf{1}_3$+$\mathbf{1}_4$/DOX, $\mathbf{1}_3$+$\mathbf{1}_4$/DOX/P-gp siRNA solution into the 6-well dish separately.
h. After 24 h incubation, collect the supernatant (floating apoptotic cells) of each well into 15 mL centrifuge tubes separately.
i. Wash the cells in each well with 1 mL of PBS.
j. Collect PBS of each well into 15 mL centrifuge tubes separately.
k. Add 0.5 mL of trypsin-EDTA to each well of the 6-well dish.
l. Gently swirl the content to cover the cell layer, then remove the trypsin-EDTA.
m. Incubate the 6-well dish in the incubator at 37°C for 3–4 min.
n. Once the cells detached, add 2 mL of the complete culture medium to inactivate trypsin.
o. Gently disperse the medium by pipetting over the cell layer surface several times.
p. Transfer the cell suspension into 15 mL centrifuge tubes separately.
q. Centrifuged (300 × *g*, 5 min, 25°C) and remove the supernatant.
r. Wash the collected cells twice with PBS (2 × 1 mL).
s. Centrifuge (300 × *g*, 5 min, 25°C) and remove the supernatant.
t. Resuspend each cell pellet in Annexin binding buffer (200 μL).
u. Add 5 μL of Annexin V-FITC into each cell suspension and stained for 15 min at 22–25°C.
v. Add 10 μL of propidium iodide (PI) into each cell suspension and stained for 15 min at 22–25°C.
w. Add the cell suspension in 5 mL round-based test tubes separately.
x. Analyze the fluorescent signals of samples on flow cytometry.

EXPECTED OUTCOMES

This protocol presents a responsive macrocycle co-delivery system that delivers drug and gene to targeted MDR cancer cells. First, we synthesized a dithiol monomer **1** using B-NHS as the starting compound (Scheme 2). After ample time to allow the thiol/disulfide exchange reaction take place and reach equilibrium, macrocycle carriers with a high drug-loading content, $\mathbf{1}_3$+$\mathbf{1}_4$/DOX nanofibers, are formed under TEM observation (Figure 2A). Through ionic interactions with siRNA, $\mathbf{1}_3$+$\mathbf{1}_4$/DOX nanofibers are subsequently kneaded into $\mathbf{1}_3$+$\mathbf{1}_4$/DOX/siRNA nanoballs (Figure 2B). $\mathbf{1}_3$+$\mathbf{1}_4$/DOX/siRNA co-delivery system exhibited enhanced cellular uptake ability (Figure 3B), as well as improved synergistic efficacy against NCI/ADR-RES cells *in vitro* (Figure 4).

LIMITATIONS

In this protocol, one potential disadvantage of the macrocycle drug and gene delivery system is that different anti-cancer drugs may affect the components of macrocycles and their structure of self-assembly. To ensure the stability of nanofiber formation, it is recommended to only use DOX as the target drug.

TROUBLESHOOTING

Problem 1

The size of white precipitate is too small, the filter paper cannot retain the precipitate (related to preparation of the reagents).

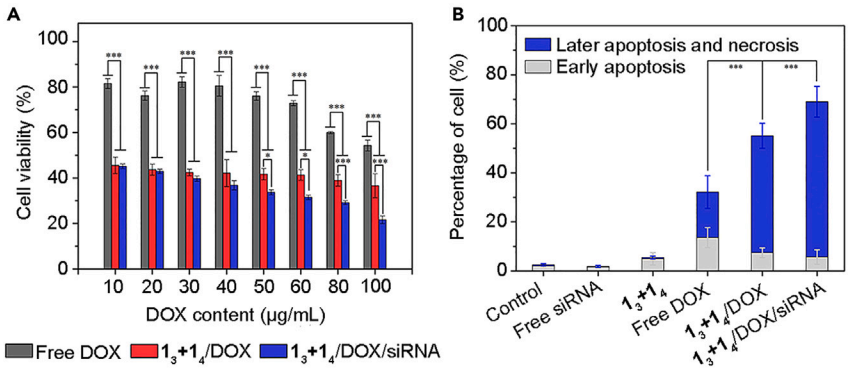


Figure 4. *In vitro* cytotoxicity and apoptosis assay

(A) *In vitro* anti-cancer efficacy. NCI/ADR-RES cells were incubated with different formulations.

(B) Percentage of NCI/ADR-RES cells in different stages from apoptosis assay after being treated with different formulations. All the measurements were performed at least in triplicate; error bars represent SDs about the mean. *P < 0.05, **P < 0.01, and ***P < 0.001. Figure reprinted with permission from Lyu et al.[1]

Potential solution

Transfer the mixture (the white precipitate and solution) into 50 mL centrifuge tubes, centrifuge (300 × *g*, 5 min, 25°C), and remove the supernatant. Wash the white precipitate with 20 mL of deionized H_2O in tubes, centrifuge (300 × *g*, 5 min, 25°C), and remove the supernatant. Resuspend the precipitate in 10 mL of deionized H_2O in tubes. Freeze-dried the solution for 48 h to afford the white pure product.

Problem 2

Low yield of 3,5-bis(tritylthio)benzoic acid (related to preparation of the reagents).

Potential solution

The low yield of 3,5-bis(tritylthio)benzoic acid (B-COOH) is due to the oxidation of 3,5-dimercaptobenzoic acid, leading to form sulfoxide byproducts. Therefore, the reactant 3,5-dimercaptobenzoic acid should be fresh prepared.

Problem 3

Poor solubility of H-Arg-NH_2 in DMF (related to synthesis of dithiol monomer 1).

Potential solution

Add H-Arg-NH_2 into a 5 mL bottom flask containing 3 mL MeOH, then sonicate until the H-Arg-NH_2 dissolve. Add the MeOH solution of H-Arg-NH_2 into the DMF solution of mixture.

Problem 4

The final product dithiol monomer **1** may be oil-like (related to synthesis of dithiol monomer 1).

Potential solution

Dissolve oil-like **1** in deionized H_2O and freeze-dried the solution for 48 h again. If the product is still oil-like, purify the product by a semi-preparative column (C18, Hypersil, 40205-259070A) on HPLC.

Problem 5

How to determine if the resuscitated NCI/ADR-RES cells are ready for the construction of stable cell lines? (related to preparation of culture cells).

Potential solution

Monitor the cell proliferation rate. The subcultivation time of the stable NCI/ADR-RES cells in the T25 cell flask is every 3–4 days.

Problem 6

The failure of siRNA transfection (related to step 3: cell uptake).

Potential solution

siRNA is extremely sensitive to RNases. To avoid RNase contamination, use the RNase-free 1.5 mL microtubes and pipette tip. As the degradation of siRNA increases with time and temperature, 1_3+1_4/DOX/siRNA nanoballs solution should be freshly prepared before transfection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jianwei Li (jianwei.li@utu.fi).

Materials availability

The reagents generated in this study will be available from the lead contact upon request.

Data and code availability

The published article (Lyu et al.[1]) includes all data generated or analyzed during this study.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.L. conceived the idea; carried out the synthesis, characterization, and cell experiments; and wrote the protocol. X.W. helped with cell experiments. J.Y. and X.W. carried out part of the synthesis. J.L. supervised the experiment, reviewed and edited the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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