SI Appendix

Photothermal Responsive Nanosized Hybrid Polymersome as Versatile Therapeutics Co-Delivery Nanovehicle for Effective Tumor Suppression

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Experimental section

Materials

The inner phase of w/o/w double emulsion droplets was made of either DOX+PVA water solution or DOX , nano-magnetite, gold nanorods, folate conjugated gold nanorods, gold nanorods conjugated porous silicon nanoparticles (cNPs) loading hydrophobic drugs, DNA + PVA solution (PVA with molecular weight 13000 to 23000, 87-89% hydrolyzed, Sigma-Aldrich). DOX, 17-AAG, Rapamycin, Erlotinib, Docetaxel, Afatinib were purchased from LC Laboratories. Experiments were conducted with the following lipids: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), fluorescent headgroup labeled phospholipid were purchased from Avanti Polar Lipids in powder form. Polycarbonate (PC) membranes at 0.2 μm and 0.4 μm and filter support were also purchased from Avanti Polar Lipids. The outer phase was 10-wt% PVA solution with 100 mOsm/L osmolarity. mPEG(5000)-b-PLA(10000) was purchased from Advanced Polymer and used without further purification. Hexane, chloroform and anti-HER2/EGFR antibody were purchased from Sigma-Aldrich. HeLa, MCF-7, SKBR3 and M28 cell lines were purchased from

American Type Culture Collection. Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), 1% penicclin-streptomycin and live/dead assay kit, Alexa Fluor 488 antibody, ATP assay kit and Human HER2 (Total) and EFGR(Full length) ELISA kit were purchased from Invitrogen Life Technologies. 96-well black clear bottom cell culture plates were purchased from Corning Inc. Solutions and solvents were filtered by 0.2 μm membrane (Anodisc or Whatman plc.) before introduction into glass capillary devices. Molecular biology grade water was purchased from Corning for Human ELISA and ATP assay and in vivo study. Highly purified distill water was acquired from a Millipore Milli-Q system.

Animals

Female nude mice (20 ± 2 g), were fed at the condition of 25 ℃ and 55% of humidity and approved by the Institutional Animal Care and Use Committee of the sixth affiliated hospital of Shanghai Jiaotong University. All animal experiments were carried out in compliance with guidelines.

Synthesis of water soluble nano magnetite and short gold nanorod

We synthesized water soluble nano magnetite according to our previous paper[1] and tested the cytotoxicity of the synthesized materials using live/dead assay. The nano magnetite is about 10 nm in diameter by TEM [1].

Gold nanorods were synthesized by seed-mediated growth. Gold seeds and short gold nanorods were prepared according to the same procedure as previously described [1,2]. The morphology of AuNRs was determined by TEM. The AuNRs-CTAB were washed three times by centrifuging at 18000 rpm for 15 min to remove excess reactants, then resuspended in Milli-Q purified water, and kept at room temperature before further experiments. The AuNRs-CTAB concentration was determined from the UV-vis spectrum (Cary 100 UV-vis spectrophotometer, Agilent Technologies)

and known extinction coefficients. AuNRs concentration or folate conjugated gold nanorods concentration in the experiments was about 10-20 nM. The concentration ratio between DOX and AuNRs is kept from 2000:1 to 10000:1.

Synthesis of dsDNA and AFM Imaging

SST AB DNA have been designed by N. Seeman [3]. All oligonucleotides were purchased from IDT, Inc. and purified by 20% denaturing PAGE. The strands of eight oligonucleotides were mixed stoichiometrically and dissolved to 0.5 μM in 1×TAE/Mg^{2+} buffer (40 mM Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM Na^{+}, 12.5 mM Mg^{2+}), 50 μL of 0.5 μM DNA in 1×TAE/Mg^{2+} buffer was slowly cooled from 95 ℃ to room temperature over 48 hours. 10 μL of SST AB DNA sample was spotted onto freshly cleaved mica, and left for 2 min, the sample drop was then washed off by 50 μL deionized water and dried by compressed air. dsDNA samples were imaged by tapping-mode AFM on Asylum-2 MFP-3D Coax AFM with NSC15 tips (silicon cantilever, MikroMasch).

Synthesis of gold nanorods conjugated porous silcon nanoparticles

The gold nanorods conjugated porous silicon nanoparticles (with COOH surface group) (AuNRsPSiNPS) were synthesized via cysteamine ($HNCHCH_2SH$) reactivated by EDC at 298 K for 24 hours under magnetic stirring. The surface COOH group of PSi NPs first reacted with amine group of cysteamine ($HNCHCH_2SH$) reactivated by EDC, then the HS group of PSi-COO-HN-$CHCH_2$-HS connected with gold nanorods. The morphology of the synthesized nanoparticles was measured by SEM and XPS.

Synthesis of folate conjugated gold nanorods and folate conjugated porous silicon nanoparticles

The folate conjugated gold nanorods were synthesized via cysteamine. Folic acid was reactivated by EDC for 30 min, then reacted with cysteamine at 298 K for 24 hours under magnetic stirring.

The COOH group of folic acid first reacted with amine group of cysteamine, then the HS group of the reactant connected with gold nanorods. The morphology of the synthesized folate conjugated gold nanorods was measured by TEM and XPS. The folate conjugated porous silicon nanoparticles were synthesized by the reaction of the COOH group of folic acid and amine group of porous silicon nanoparticles. The COOH group of folic acid was reactivated by EDC for 30 mins, then reacted with the surface amine group of porous silicon nanoparticles under magnetic stirring for 24 hours at 313 K. The synthesized nanoparticles were obtained after 18000 rpm for 15 min.

Generation of w/o/w Double emulsion

We use glass capillary microfluidic devices to produce monodisperse hybrid vesicles with a shell composed of phospholipid (either DOPC or POPC) and mPEG(5000)-b-PLA(10000), and a core containing the water-soluble anticancer drug DOX, DNA, anti-HER2/EGFR antibody, nano-magnetite, gold nanorods, folate conjugated gold nanorods, gold nanorods conjugated porous silicon nanoparticles co-loading hydrophobic drugs Afatinib and Docetaxel and/or Rapamycin. We also generate the pure polymersomes with a shell of mPEG(5000)-b-PLA(10000) and a core containing gold nanorods and nano magnetite using double emulsions as templates. The round capillaries with inner and outer diameters of 0.58 μm and 1.0 mm were purchased from World precision instruments, Inc., and tapered to desired diameters with a micropipette puller (P-97, Sutter Instrument, Inc.) and a microforge (Narishige international USA, Inc.). The tapered round capillaries were fitted into square capillaries (Atlantic International Technology, Inc.) with an inner dimension of 1.0 mm for alignment. The outer diameter of the double emulsion varied from 30 to 50 μm, while the inner diameter varied from 20 to 30 μm. These values were controlled by the size of the capillaries used and the flow rates of different phases. The typical flow rates for generating w/o/w emulsion for the inner, middle and outer phase were 500, 1100,

and 2500 μL/hr, respectively, and the double emulsion droplets generation frequency was about 500 Hz. The total diblock copolymer and phospholipid concentration in the middle oil phase was controlled from 6-10 mg/mL and 2 mg/mL, respectively. The concentration of DOX in the inner water phase was up to 10 mg/mL and 17-AAG or Afatinib or Docetaxel or Rapamycin in the middle phase was up to 10 mg/mL. The nanosized droplets could be fabricated directly through the extrusion of the double emulsion droplets formed on microfluidics chip via different sizes of filter (0.2 μm and 0.45 μm).

Characterization of the multifunctional hybrid polymersomes.

Colorful, bright field, phase contrast and fluorescence images were obtained with 60× and 10× objectives at room temperature using Nikon Eclipse TE2000-E or inverted fluorescence confocal microscope (Leica, DMIR or DMIRBE) with a high speed camera (Phantom v7.3, v7, and v5) or digital camera (QImaging, QICAM 12-bit). All double emulsion generation processes were monitored with the microscope either with high speed camera or digital camera. The hybrid polymersomes with and without magnetic nanoparticles and gold nanorods conjugated porous silicon nanoparticles were imaged by Nikon TE-2000E colorful and Leica confocal microscope. The morphology of the nanosized hybrid polymersomes was studyed by Cryo-TEM (FEI Tecnai Arctica CryoTEM with Autoloader).

The stability study of different hybrid polymersomes.

We did assess the stability of the hybrid polymersomes in the absence of cells. In order to compare the stability of pure polymersome and the hybrid polymersomes with different ratio of lipid, we have left the pure polymersomes and hybrid polymersomes in the aqueous solution with same osmolarity of 100 mOsm/L and quantify the remaining number every 7 days for one month using microscopy, and we concluded that the stability of the hybrid polymersomes is decreased

with lipid content. The original number of the different polymersomes is 30, after one month, only 20 left for 10:3 hybrid polymersomes, 25 left for 10:2 hybrid polymersomes, 27 left for 10:1 hybrid polymersomes, and 30 left for 10:0 pure polymersomes.

Drug encapsulation efficiency determination.

We encapsulated water soluble anticancer drug DOX in multifunctional hybrid polymersomes formed either by the slow evaporation of solvent by dialysis through an anodized alumina filter as the dialysis membrane or directly formed in the glass slide by dewetting. We measured the initial concentration of DOX in the inner water phase and in the collected w/o/w emulsion by UV spectrum (NanoDrop ND-1000 Spectrophotometer) at 488nm, the standard curve of DOX was measured at 488 nm by UV Spectrum. The drug encapsulation efficiency was calculated based on the percentage of real encapsulated drug concentration and initial drug concentration.

The concentration of 17-AAG (Docetaxel or Rapamycin) or Afatinib was analyzed by injecting 20 μL of each diluted sample into Agilent 1200 HPLC/MDS Sciex 3200 Q-trap tandem MS system in negative electro-spray mode for quantification using water and acetonitrile as a mobile phase.

The concentration of Alexa Fluor 488 antibody was analyzed by fluorescent intensity at 488 nm emission by microplate reader SpectraMax M2 Molecular device). We encapsulated the certain amount of either fluorescent antibody (Alexa Fluor 488 antibody) or fluorescent antibody (Alexa Fluor 488 antibody) loaded cNPs into the hybrid polumersomes on microfluidics chip, then fabricated the two different fluorescent stained nanovehicles using extrusion method, we could measure the fluorescence intensity of fluorescence antibody solution, two hybrid polymersomes suspensions and two stained nanovehicle suspensions using microplate reader. Based on the fluorescence intensity data from the microplate reader, we could calculate the encapsulation

efficiency of fluorescent antibody and fluorescent antibody loaded cNPs in hybrid polymersomes and nanovehicles. The calculated encapsulation efficiency of the fluorescence antibody loaded cNPs by the hybrid polymersomes and nanovehicles is about 96.3 ± 3.5 % and 92.6 ± 3.3 % respectively.

The therapeutics loading content was calculated based on the weight ratio between the drug and hybrid vesicles.

In vitro dynamic release.

In vitro release of free DOX water solution, DOX, Afatinib, Docetaxel, antibody, 17-AAG, DNA, DOX+DNA, DOX+Afatinib and DOX+DNA+Afatinib loaded multifunctional hybrid vesicles PBS suspensions were evaluated by a dynamic dialysis method. Aliquot of each drug-loaded PBS solution (0.8-1 mL) was placed in the mini dialysis kits (2 mL, MWCO 8kDa cut-off) (GE Health, USA) and floated in 100 mL PBS +0. 1 wt % Tween 80 release medium and maintained at 37 ^{o}C with a paddle revolution speed of 100 r/min using G24 Environmental incubator shaker (New Brunswick Scientific Co. Inc.). At intervals, 500 μL of release sample was withdrawn and 500 μL fresh release medium was added to the bulk solution. The concentration of (Docetaxel, Rapamycin or 17-AAG) or Afatinib from the release sample was analyzed by LC-MS (Agilent 1200 LC/MDS Sciex 3200 Q-trap tandem MS system). The concentration of DOX was measured and calculated from the maximum peak by UV-VIS spectrum at 488 nm (ND-1000 nanodrop or Cary Agilent 1100). The concentration of antibody from the release samples were analyzed by microplate reader at 488nm excitation wavelength.

Photothermal effects induced by laser irradiation of gold nanorods

The photothermal effects induced by both thermal heating and laser-induced heating of gold nanorods has been performed under laser irradiation at 650 nm or 808 nm wavelength from 5 to

30 minutes respectively. The release of DOX and gold nanorods from cNPs multifunctional hybrid polymersomes was measured and calculated by UV-Vis spectrum (Cary Agilent 60) at a maximum UV absorbance of 488 nm and at a maximum UV-vis absorbance of 930 nm wavelength.

Cell cytotoxicity

We did the cell cytotoxicity according to our previous work [1,2]. HeLa (a human cervical carcinoma cell line), MCF-7 (human breast carcinoma line), SKBR-3 (HER2 postive breast cancer cell line) and M28 (Human mesothelioma cell line) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicclin-streptomycin (PS). The cell medium was filtered through 0.2 μm filter. SKBR-3/AR is the afatinib resistant SKBR-3 cells, it is the SKBR-3 cells treated by 1 ng/mL Afatinib and incubated in an atmosphere of 5% CO_2 at 37°C for 1 month. The survival cells after Afatinib treatment is the SKBR-3/AR cells.

Live and dead assay: 100 μL of 1 μM Calcein AM + 2 μM of EthD-1 culture medium were added to 100 μL cell medium containing either HeLa, MCF-7 cells, SKBR3, MCF-7/DOX or M28 cells with or without multifunctional hybrid vesicles seeded on 96-well culture plates at a density of 1×10^4 cell/mL in an atmosphere of 5% CO_2 at 37°C and cultured for 1 to 24 h. We counted the cell number and density by using Invitrogen Countess automated cell counter. The fluorescence intensity was measured at the excitation wavelength of 488 nm excitation for Calcein AM and 544 nm for EthD-1, and the emission wavelength was 530 nm for Calcein AM and 610 nm for EthD-1 using a microplate reader SpectraMax M2 (Molecular device). The cell viability of blank and multifunctional hybrid vesicles suspensions on HeLa, MCF-7, SKBR3, MCF-7/DOX or M28 cell lines was calculated based on the negative control, blank control and samples fluorescence intensity according to live/dead assay using the following equation:

$$\% \text{ Live Cells} = \frac{[F(530\text{ nm})_{sample} - F(530\text{ nm})min]}{[F(530\text{ nm})_{max} - F(530\text{ nm})_{min}] \times 100\%.}$$

We use two-colored Apoptosis/Cytotoxicity Demo kit from Essen BioScience and Essen's CellPlayer HeLa NucLight Red cell lines to study the cytotoxicity of the formed hybrid vesicles at different concentration according to the protocols by the manufacture. We seeded a 96-well plate with 2500 cells/well and immediately placed the 96-well plate in IncuCyte ZOOM (ESSEN BIOSCIENCE) and set the scan schedule every hour using multiple channel phase (red and green) at 37 ^{o}C. HeLa NucLight Red cell was maintained in F-12 culture medium containing 10 % FBS and 1% P/S.

ATP detection kit cytotoxicity assay

The ATP determination kit (A22066, Life Technologies) standard protocol was used to carry out the experiment towards HeLa cells seeded on 96 well culture plates on density of 10^4 cells per well at 37 ^{o}C for 4 hour after treatment of drug, DNA, drugs, drug/drugs with DNA combination. The luminescent intensity of ATP standards and treated samples was measured at 560 nm emission using SpectraMax L plate reader (Molecular device).

Human HER2 (Total) ELISA Assay

The Invitrogen Human HER2 (Total) ELISA Kit was used to detect and quantify the full-length HER2 protein independent of its phosphorylation state from HER2 positive breast cancer cell SKBR-3 lysates treated with different therapeutics. SKBR-3 cells with density of 10^6 /mL were seeded on 12 well cell culture plate and treated with different therapeutics for 6 hours. The optical density at 450 nm of the standards and samples according to the assay kit standard protocol was obtained using microplate reader (SpectraMax i3 from Molecular device).

Human EGFR (Full length) ELISA Assay

The Invitrogen Human EGFR (Full length) ELISA Kit was used to detect and quantify the full-length EGFR protein independent of its phosphorylation state from EGFR positive cancer cell

HeLa lysates treated with different therapeutics. HeLa cells with density of $5x10^6$ /mL were seeded on 12 well cell culture plate and treated with different therapeutics for 6 hours. The optical density at 450 nm of the standards and samples according to the assay kit standard protocol was obtained using microplate reader (SpectraMax i3 from Molecular device).

in vivo anti-tumor study

SKBR-3 HER2 positive breast tumor-bearing nude mice were treated with free multifunctional nanovehicle, single drug-nanovehicle and drugs (weight ratio 1:1) combination-nanovehicle at the total dosage of 5 mg drug/kg mouse or 2.5 mg/kg for double and triple drug combination through intravenous injection. Each group contains six mice. The formulations were intravenously injected on days 0, 2, 4, …and 28, 2 days per injection for 4 weeks. The tumor sizes were measured every two days, and the volume was calculated according to the formula: $V = 0.5 \times a \times b^2$, where a was the length of the tumor and b was the width of the tumor. The mice were weighed, and survival was recorded. The tumor was peeled off from the mice at the end of the treatment and the tumor suppression rate was calculated based on the volume of the tumor.

Histological Assessment

For the histological analysis (TUNEL staining), HER2 positive breast tumor-bearing mice were sacrificed at day 28 after the first treatment, and the tumors were collected and fixed in 10% formalin and embedded in paraffin blocks to prepare tumor sections at a thickness of 5 μm. After deparaffinization, the tissue sections were stained with hematoxylin and eosin (H&E) and terminal deoxynucleotidyltransferase mediated UTP end labeling (TUNEL) and were visualized by a fluorescent optical microscope (Olympus, Japan). The bcl-2 protein and Caspase 3 protein expression were investigated by immunocytochemistry from paraffin-embedded tissue in mice with breast cancer stained with blue and brown fluorescent dyes, which were visualized by a

fluorescent optical microscopr (Olympus, Japan). EGFR and VGFE protein expression ELISA were carried out for PBS control, blank nanovehicle, single drug, two and three drugs combination according to standard protocol. The fluorescence in vivo bioimaging of free Cy-7 siRNA and nanovehicle-Cy7 siRNA in tumor-bearing nude mice through intravenous injection was visualized at 2h, 8h, 24h and 48h by a fluorescent optical microscope (Olympus, Japan).

*Statistical analyse*s

All data represent mean ± S.D. (n = 3). The data were analyzed by a Student's t-test using the online version of GraphPrism software. The significance was set at probabilities of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Supporting figures

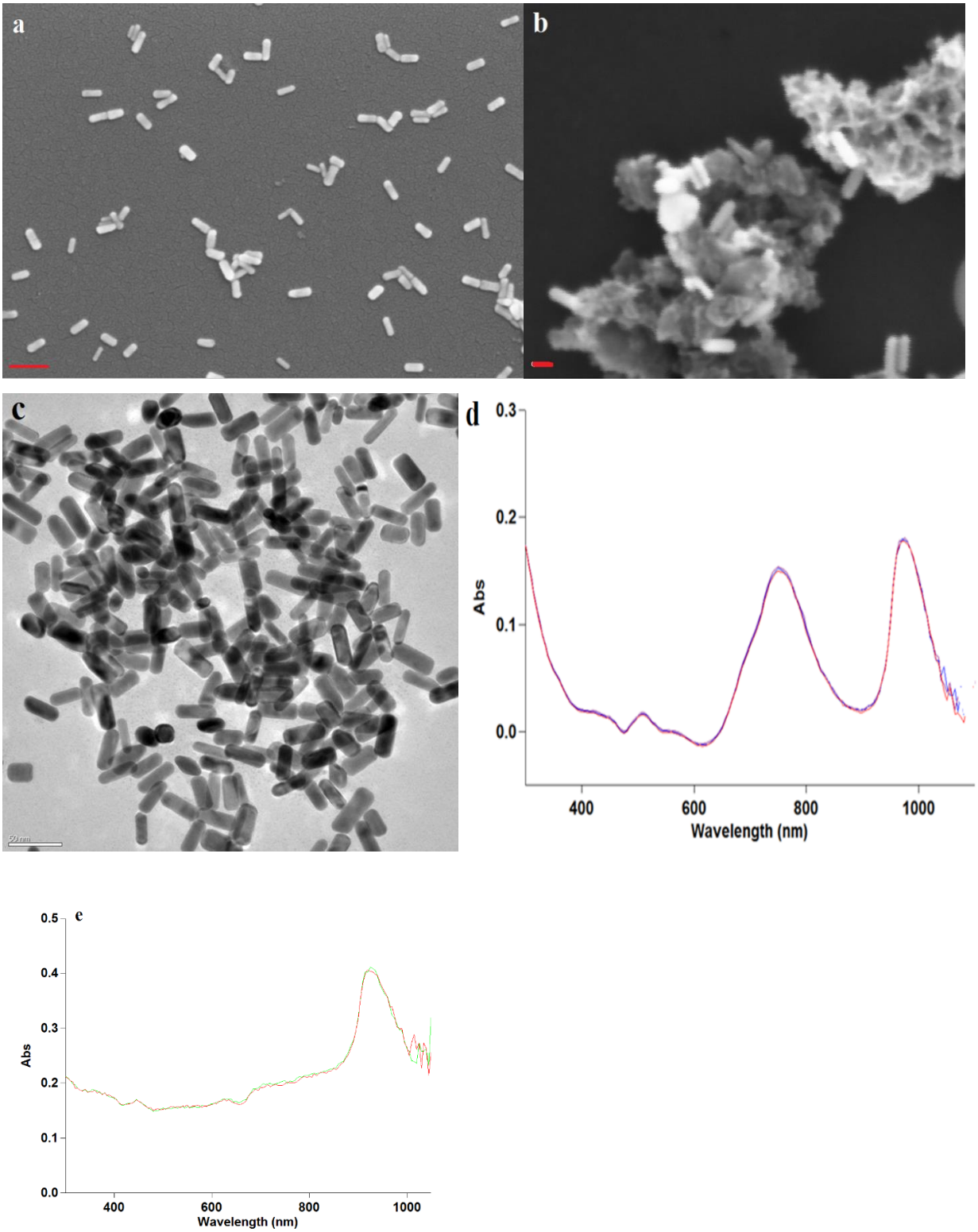
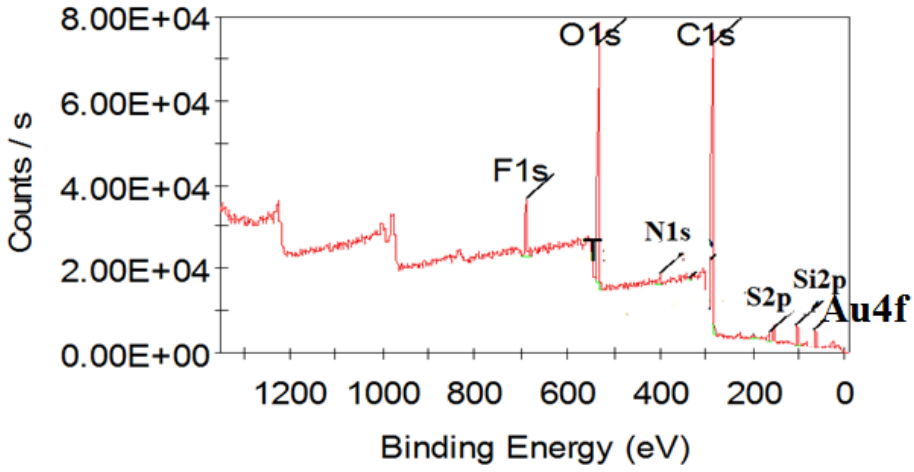


Figure S1. Morphology of the prepared gold nanorods and UV-vis measurement.

(a)SEM image of gold nanorods (AuNRs); the scale bar denotes 100 nm. (b) SEM image of porous silicon nanoparticles conjugated gold nanorods (cNPs); the scale bar denotes 20 nm. (c)TEM image of folate conjugated gold nanorods. The scale bar denotes 50 nm. (d)The UV-vis spectrum of folate conjugated gold nanorods. (e)UV-vis spectrum of gold nanorods conjugated porous silicon nanoparticles.



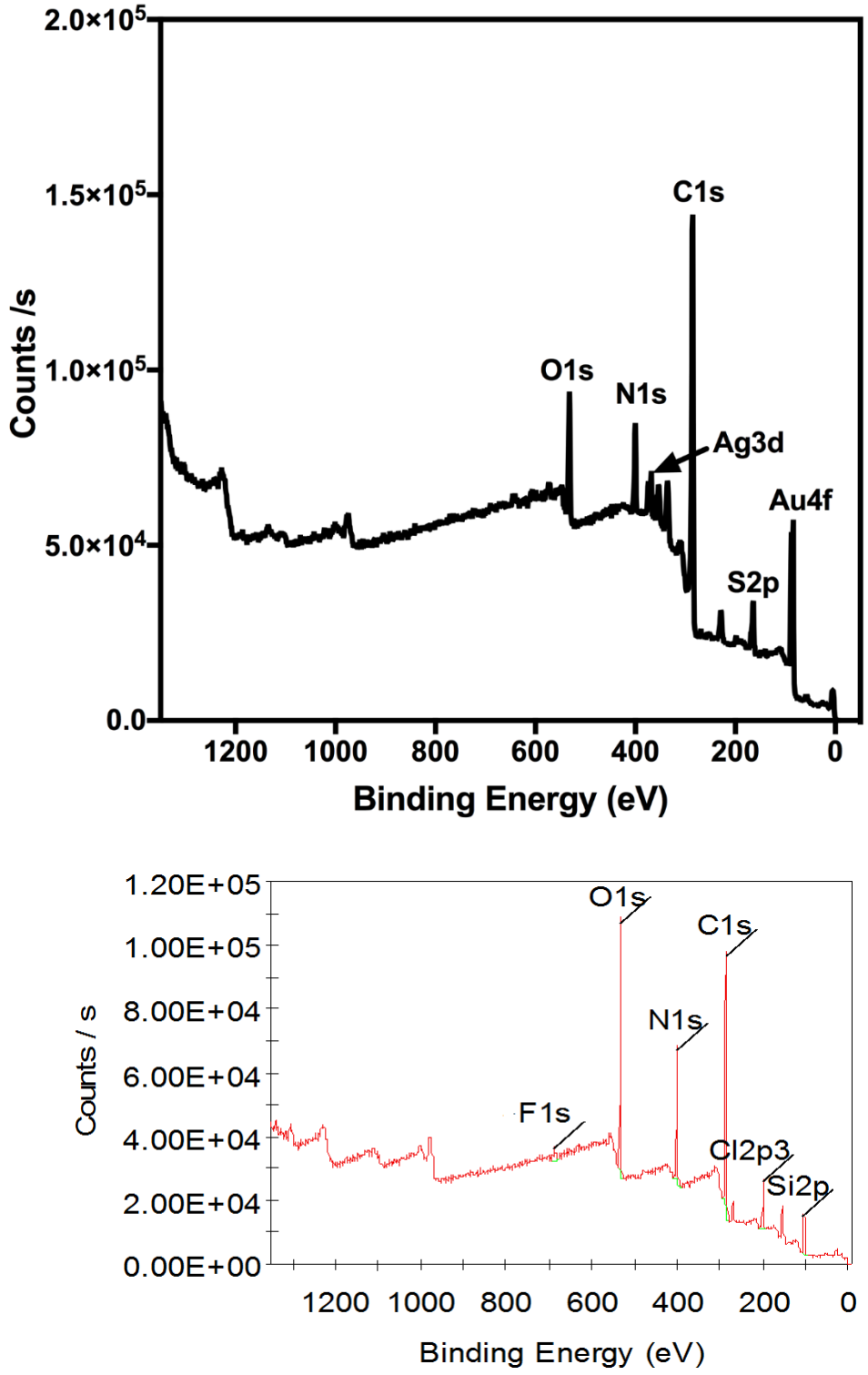


Figure S2. The X-ray photoelectron spectroscopy (XPS) of the gold nanorods conjugated porous silicon nanoparticles (cNPs), folate conjugated gold nanorods, and folate conjugated porous silicon nanoparticles.

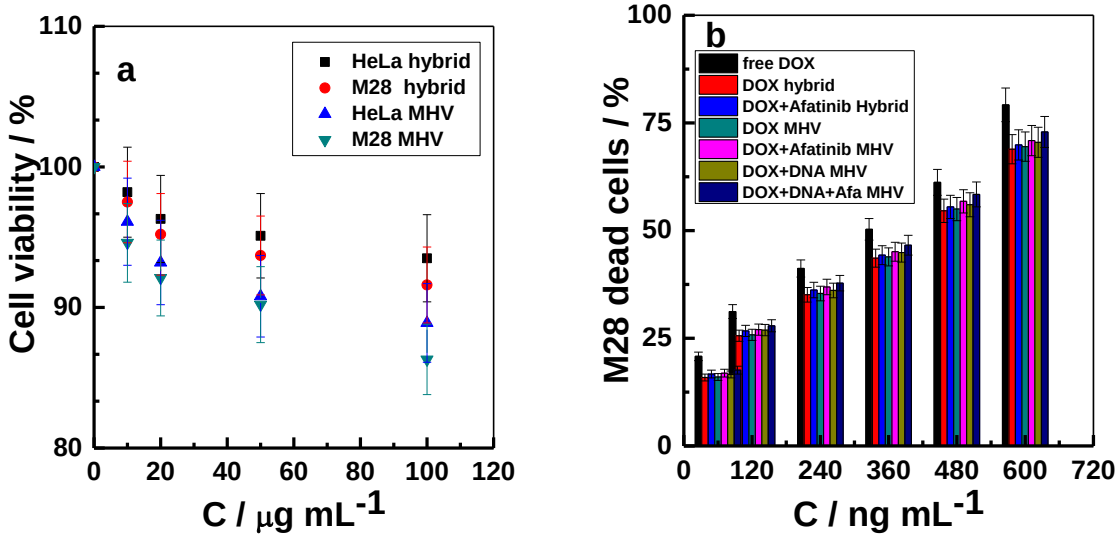


Figure S3. In vitro cell cytotoxicity and side effect reduction of DNA and multifunctional hybrid vesicles (MHV).

(a)HeLa and M28 cell viability in the presence of hybrid vesicles, antibody and DNA multifunctional hybrid polymersomes (MHV) suspensions (10 μg/mL nano-magnetite, 10 nM folate conjugated gold nanorods and 400 μg/mL cNPs) at different concentration after 24 h incubation at 37 ^{o}C. Error bars represent standard deviation of the three independent experiments in triplicate wells of cells.

(b) Side effect reduction of multifunctional hybrid vesicles on M28 cells after 24 h incubation at 37 C using live/dead assay. The multifunctional hybrid vesicles (MHV) with nano-magnetite and gold nanorods on HeLa and MCF-7 cells after 24 h incubation ($C_{DOX}/C_{Afatinib}$ = 2:1; the concentration of gold nanorods and nano-magnetite is about 10 nM and 10 μg/ mL). (MHV with 10 μg/ mL nano-magnetite, 10 nM folate conjugated gold nanorods and 400 μg/mL cNPs set as control). Error bars represent standard deviation of the three independent experiments in triplicate wells of cells.

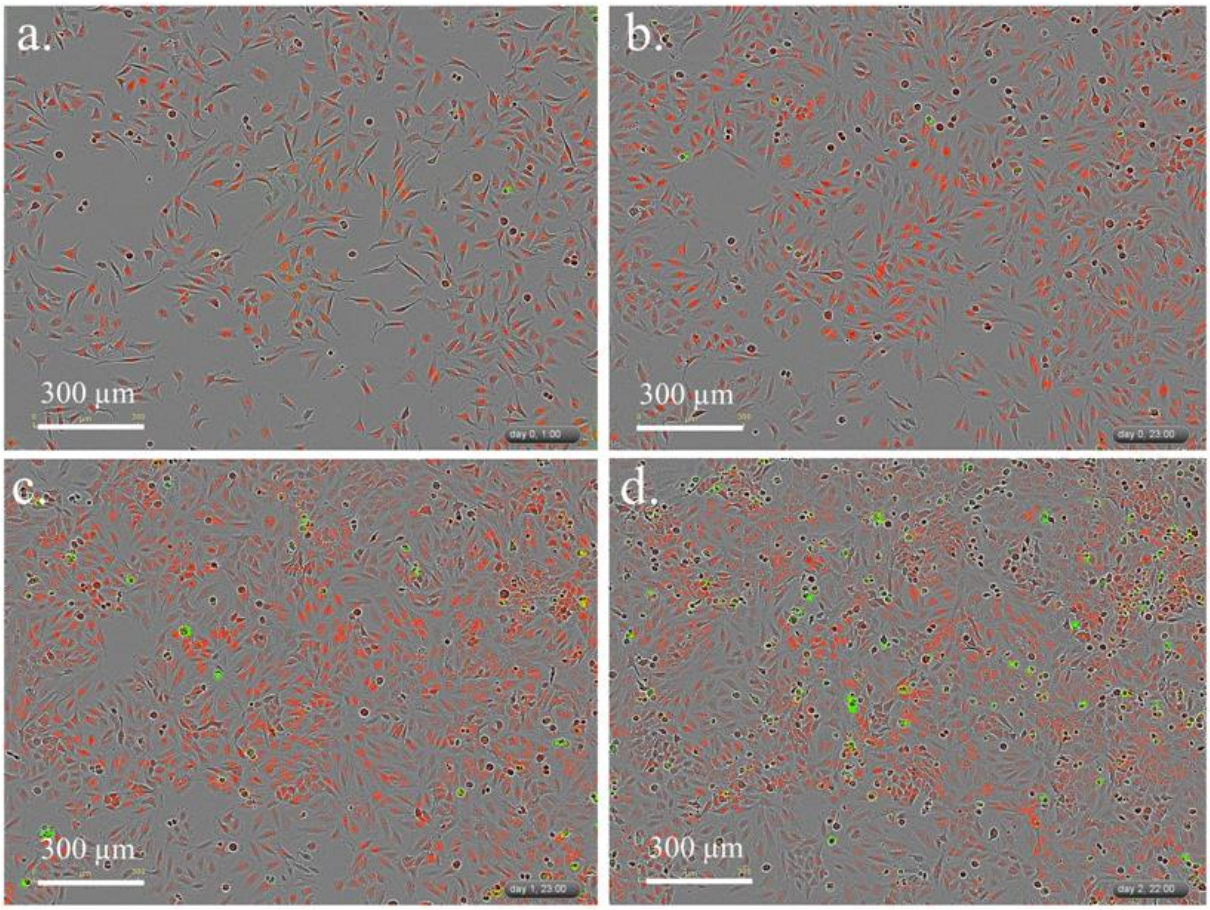


Figure S4. *In vitro* cytotoxicity study.

(a-d) Cytotoxicity of the formed gold nanorods conjugated porous silicon nanoparticles (cNPs) and folate conjugated gold nanorods functionalized hybrid polymersomes on HeLa NucLight Red cells incubated from 1h to 48 at 37 ^{o}C using 2-color Cytotoxicity Demo Kit.

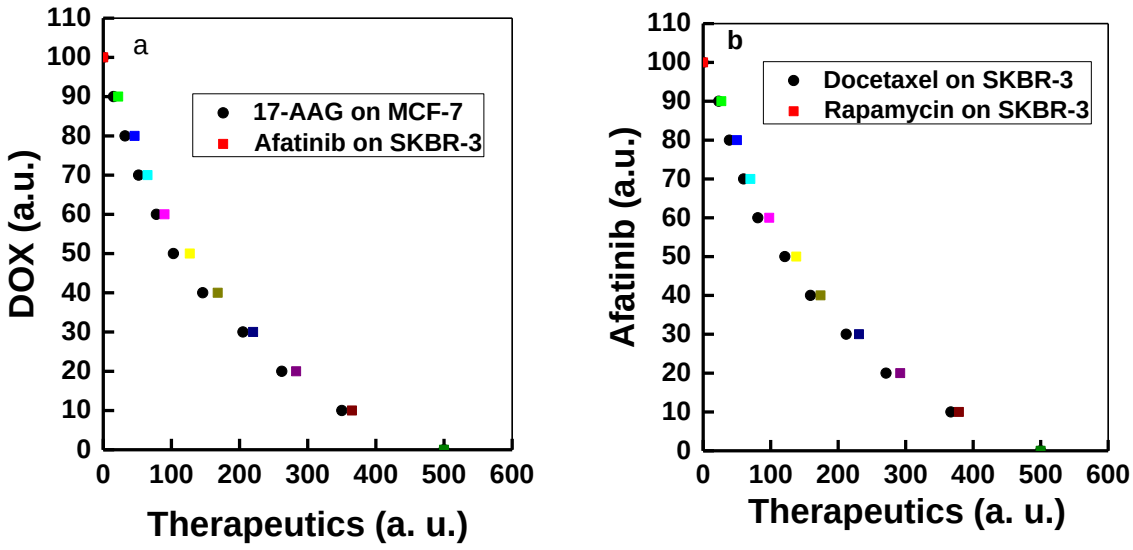


Figure S5. In vitro synergism study.

Isobologram. (a) Abscissa and ordinate units are the concentrations of the drugs DOX and 17-AAG on MCF-7 cells, DOX and Afatinib on SKBR-3 cells; the unit of DOX is 100, and the unit of 17-AAG or Afatinib is 500. (b) Abscissa and ordinate units are the concentrations of the drugs combination of either Afatinib and Docetaxel or Afatinib and Rapamycin on SKBR-3 cells; the unit of Afatinib is 100, and the unit of Docetaxel or Rapamycin is 500.

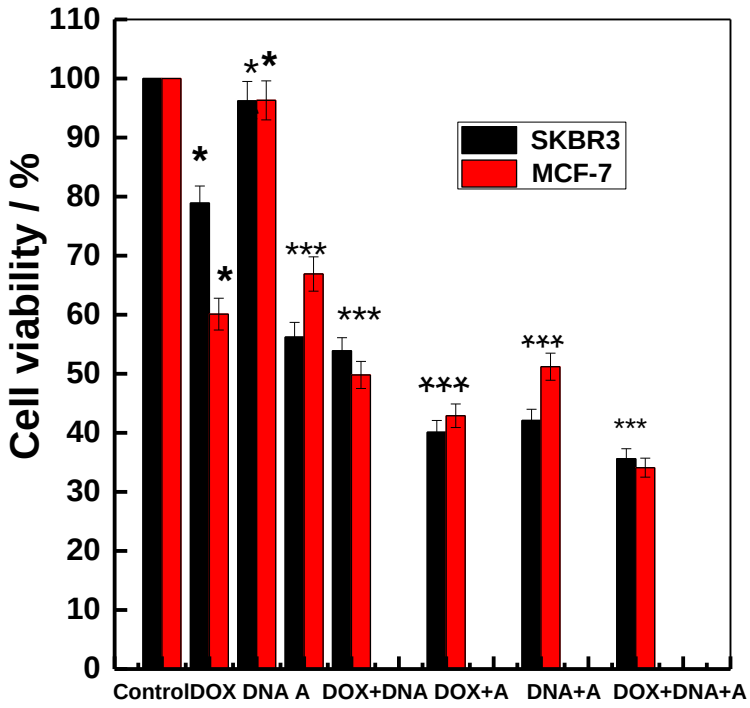


Figure S6. Synergistic cell killing effect by drugs combination and DNA drugs combination on SKBR3 and MCF-7 cells after 24 h incubation ($C_{DOX}/C_{Afatinib}$ = 2:1; the total concentration of the single drug and multiple drugs is kept at 200 ng/mL ; DNA 10 nM; and multifunctional hybrid vesicles co-loaded with 10 μg/mL nano-magnetite, 10 nM folate conjugated gold nanorods and 400 μg/mL cNPs set up as control) using live/dead assay. The data were analyzed by a Student's *t*-test using the online version of Graph Prism software with the significance probabilities of ***$p < 0.001$.

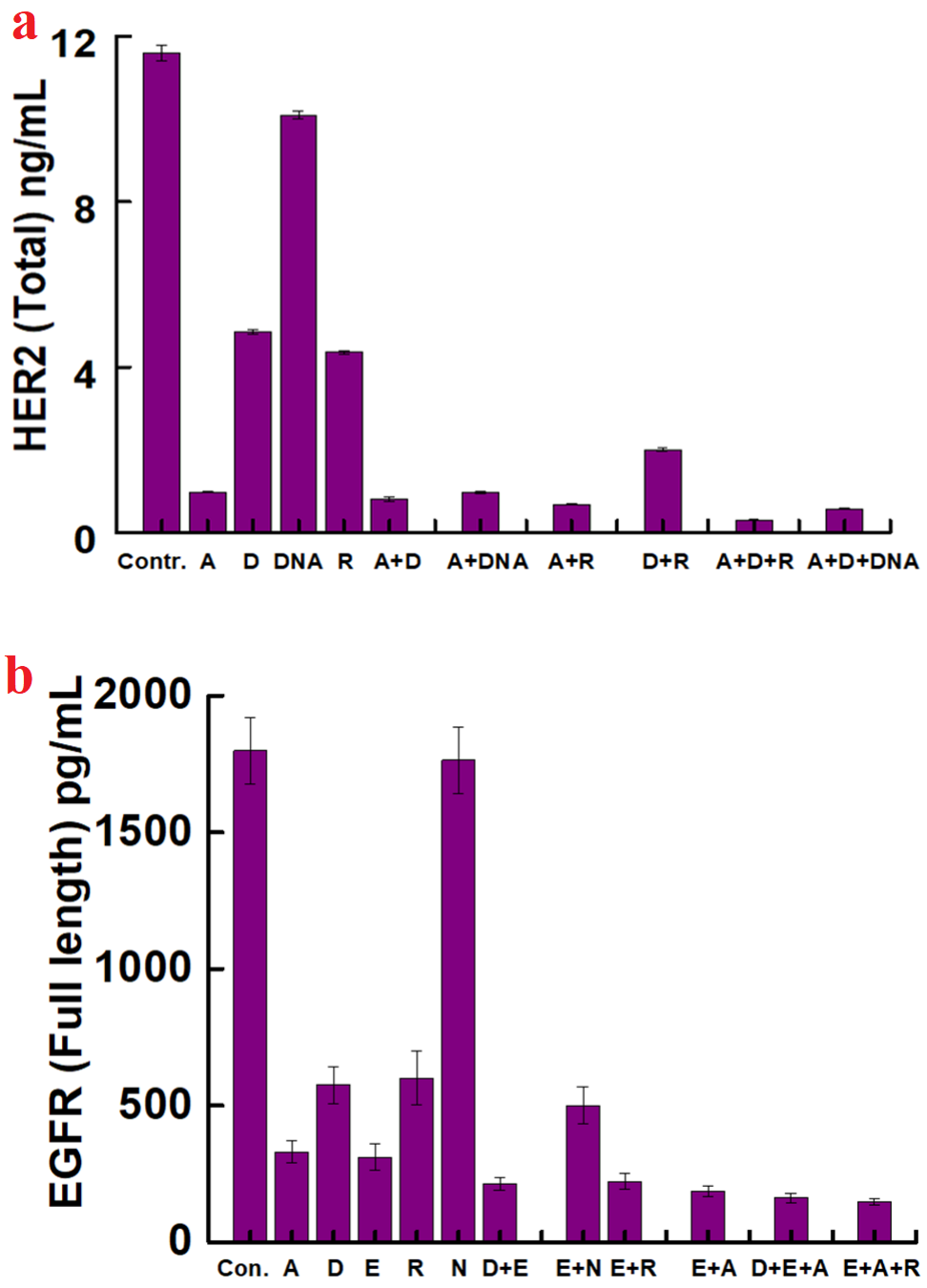


Figure S7. The Human HER2 (Total) and EGFR (Full length) ELISA study for measurement of HER2 and EGFR protein at optical density 450 nm on SKBR-3 cells and HeLa cells. Afatinib (A), Erlotinib (E), Rapamycin (R), Docetaxel (D), DNA (N). The total drug concentration is 9 µg/mL; C_D/C_E =1:1; C_D/C_R =1:1; C_A/C_E =1:1; $C_A/C_D/C_E$= 1:1:1; DNA is 10 nM; MHV with 10 µg/mL nano-magnetite, 10 nM folate conjugated Au NRs and 400 µg/mL cNPs set up as control). All data represent mean ± S.D. (n = 3).

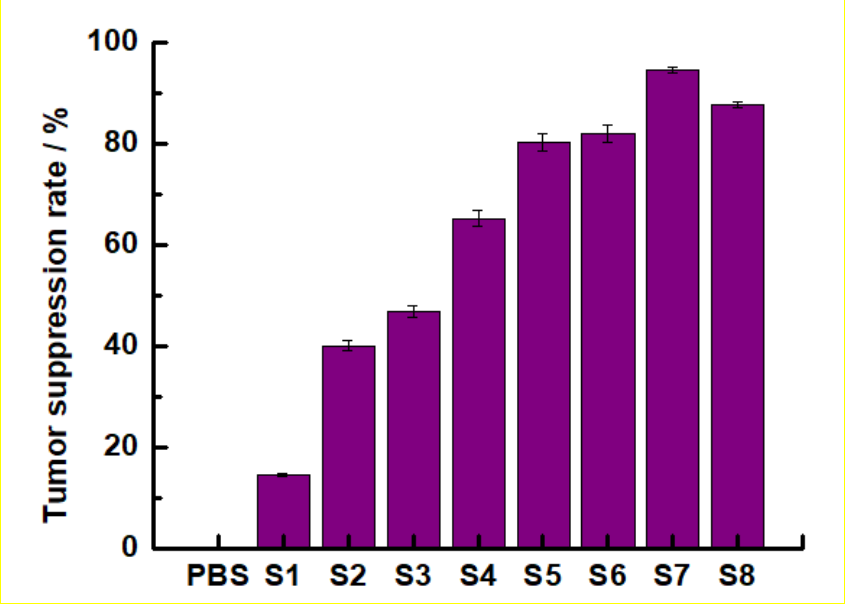


Figure S8. The tumor suppression rate once every 48 hours for 4 weeks intravenous injection treatments (six mice per group) by PBS, S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@nanovehicle at total dosage of 2.5mg/kg).The total dosage from S2 to S7 is 5 mg/kg. The drug ratio for two and three drug combinations is 1:1. Each group contains six mice.

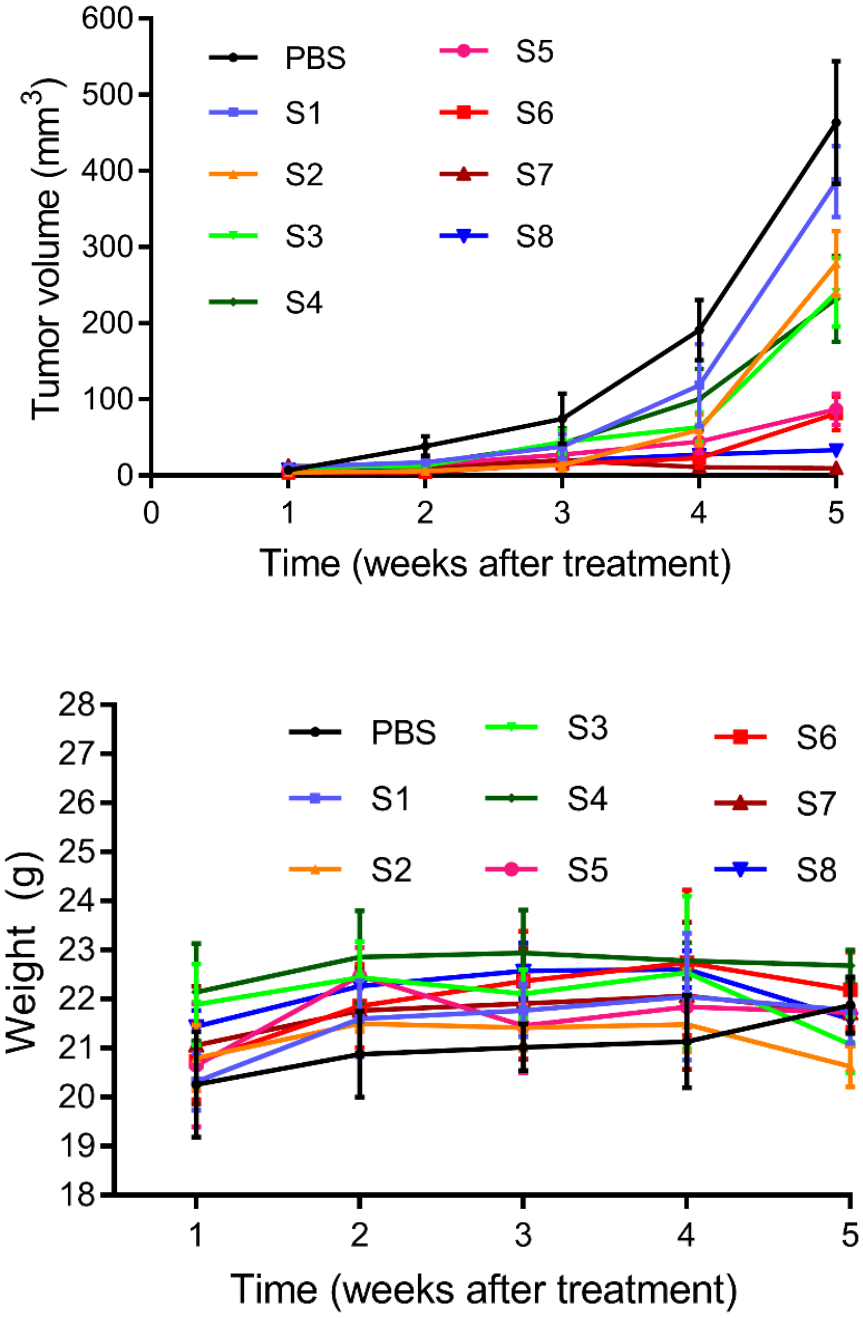


Figure S9. The tumor growth curves and body weight changes with intravenous injection treatment time.

(a)The tumor volume curves after different intravenous injection treatment. (b) The mice weight changes with intravenous injection treatment time. PBS, S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+ Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@nanovehicle at total dosage of 2.5mg/kg).The total dosage from S2 to S7 is 5 mg/kg. The drug ratio for two and three drug combinations is 1:1. Each group contains six mice.

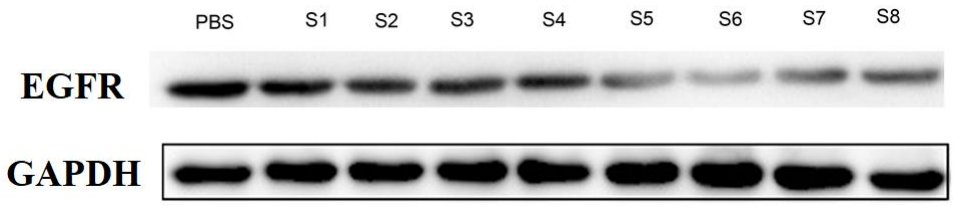


Figure S10. The EGFR protein expression after treatment by PBS, S1 (nanovehicle), S2(Docetaxel@nanovehicle), S3(Rapamycin@nanovehicle), S4(Afatinib@nanovehicle), S5(Docetaxel+Afatinib@nanovehicle); S6(Rapamycin+ Afatinib@nanovehicle), S7(Docetaxel+Rapamycin+Afatinib @nanovehicle), S8(Docetaxel+Rapamycin +Afatinib @nanovehicle), The total dosage of S2 to S7 for intravenous injection is 5 mg /kg; the total dosage of S8 is 2.5 mg/kg. GAPDH is set up as control.

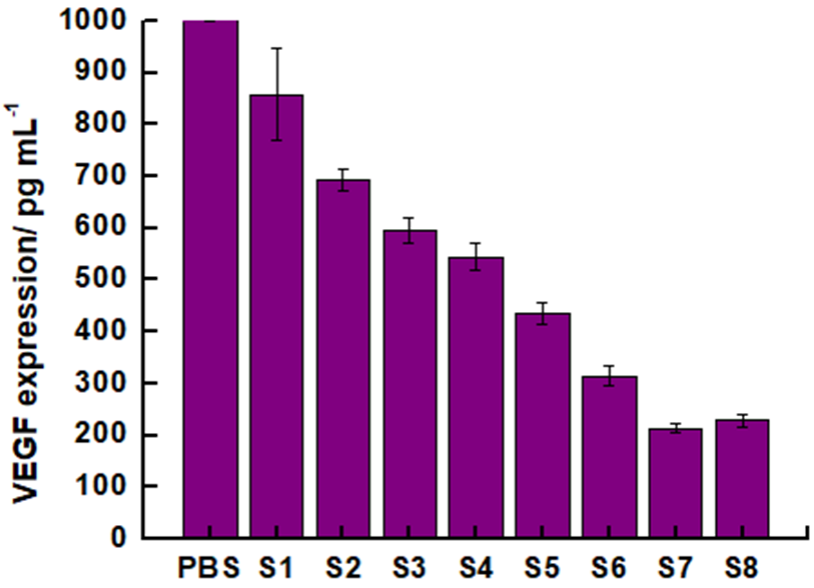


Figure S11. The VEGF protein expression after treatment by PBS, S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@nanovehicle at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.

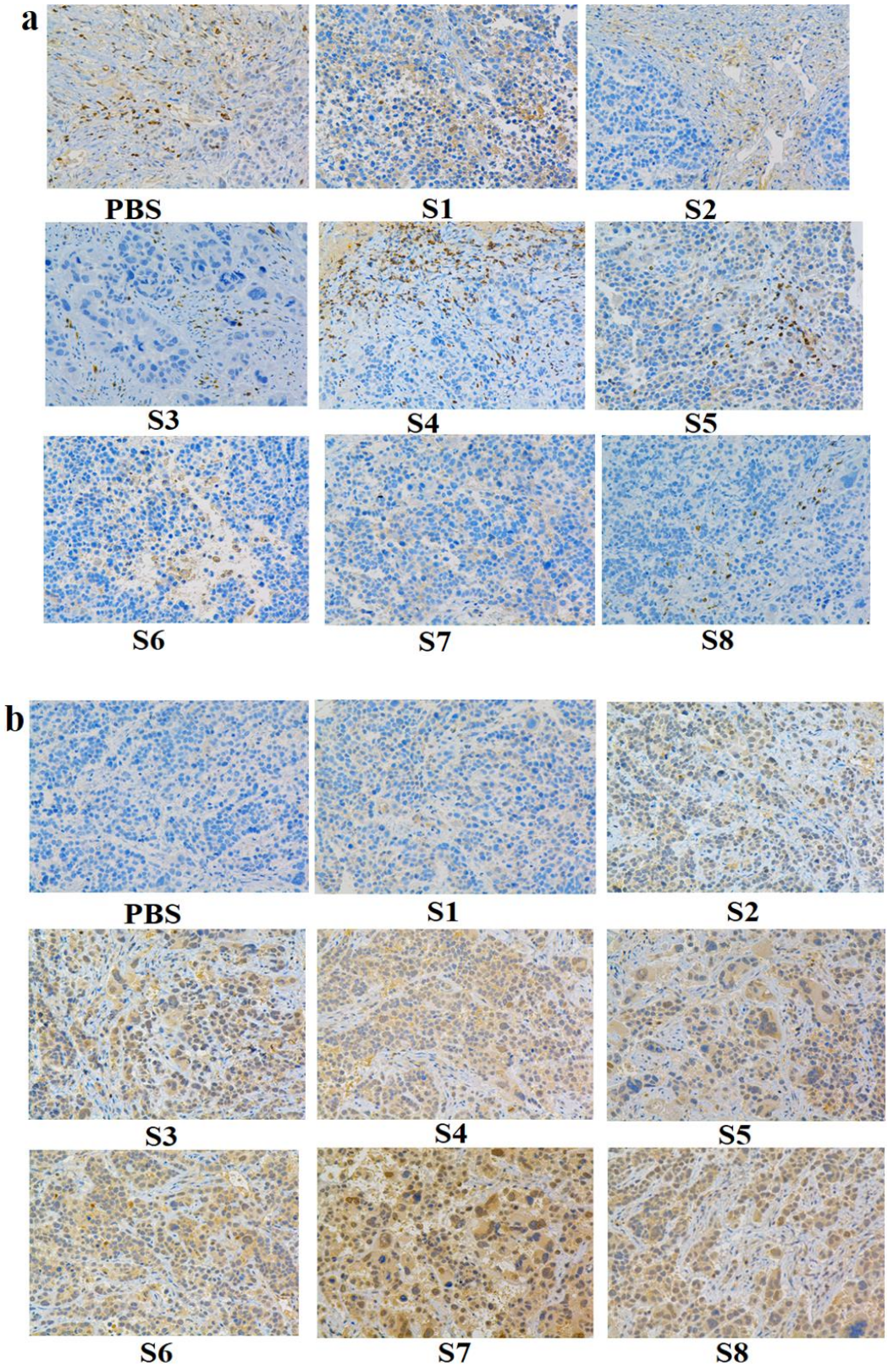


Figure S12. Bcl-2 protein expression and Caspase 3 protein in vivo study.

(a)Bcl-2 protein expression study after different system treatment. PBS, S1 (blank nanovehicle), S2 (D-NV), S3 (R-NV), S4 (A-NV), S5 (D+A-NV), S6 (R+A-NV), S7 (D+R+A-NV), S8 (D+ R +A-NV at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.

(b)Caspase 3 protein expression study. PBS, S1 (blank nanovehicle), S2 (D-NV), S3 (R-NV), S4 (A-NV), S5 (D+A-NV), S6 (R+A-NV), S7 (D+R+A-NV), S8 (D+R+A-NV at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.

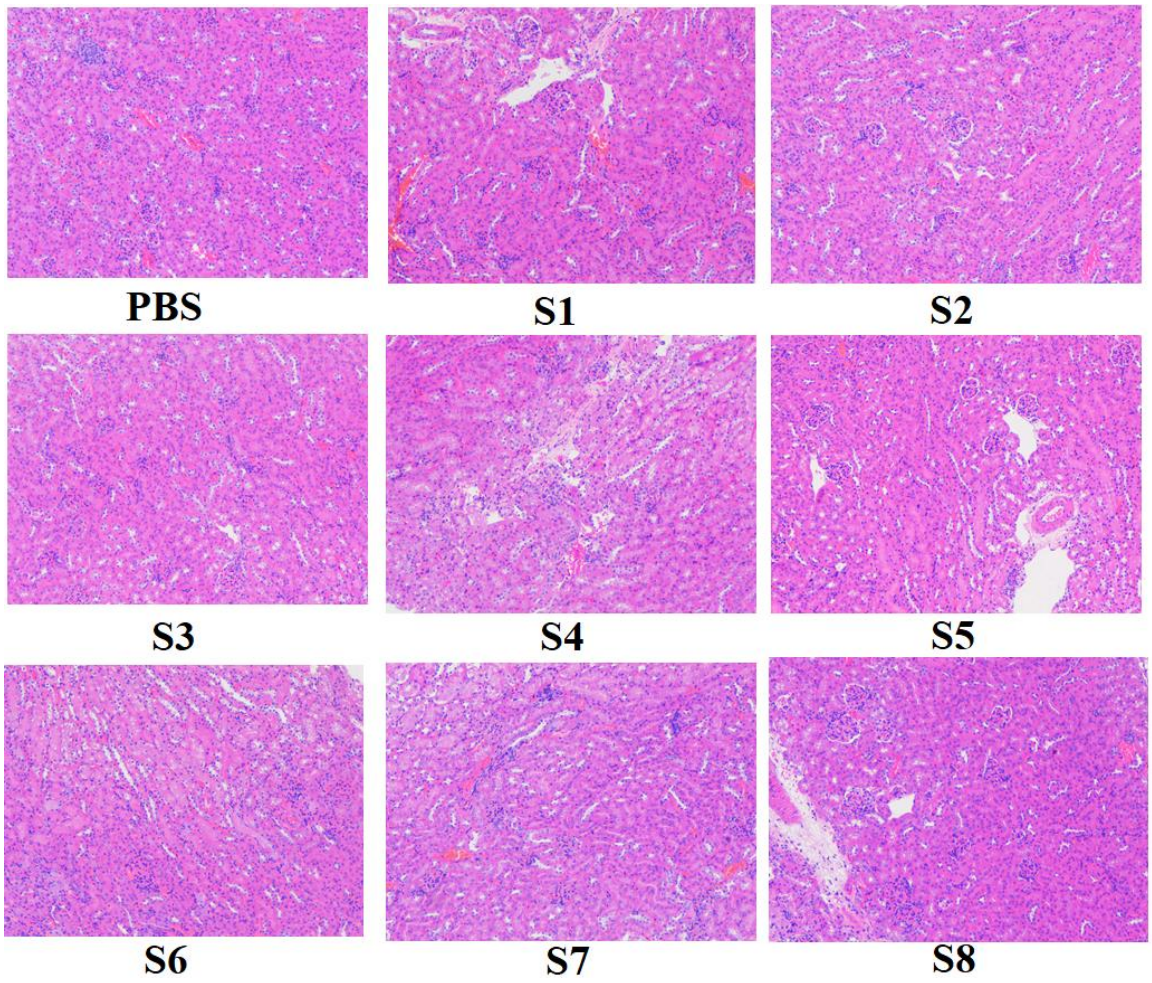


Figure S13. Hematoxylin and eosin (HE) stain of kidney. The mice was treated with S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin + Afatinib @ nanovehicle at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combination is 1 to 1.

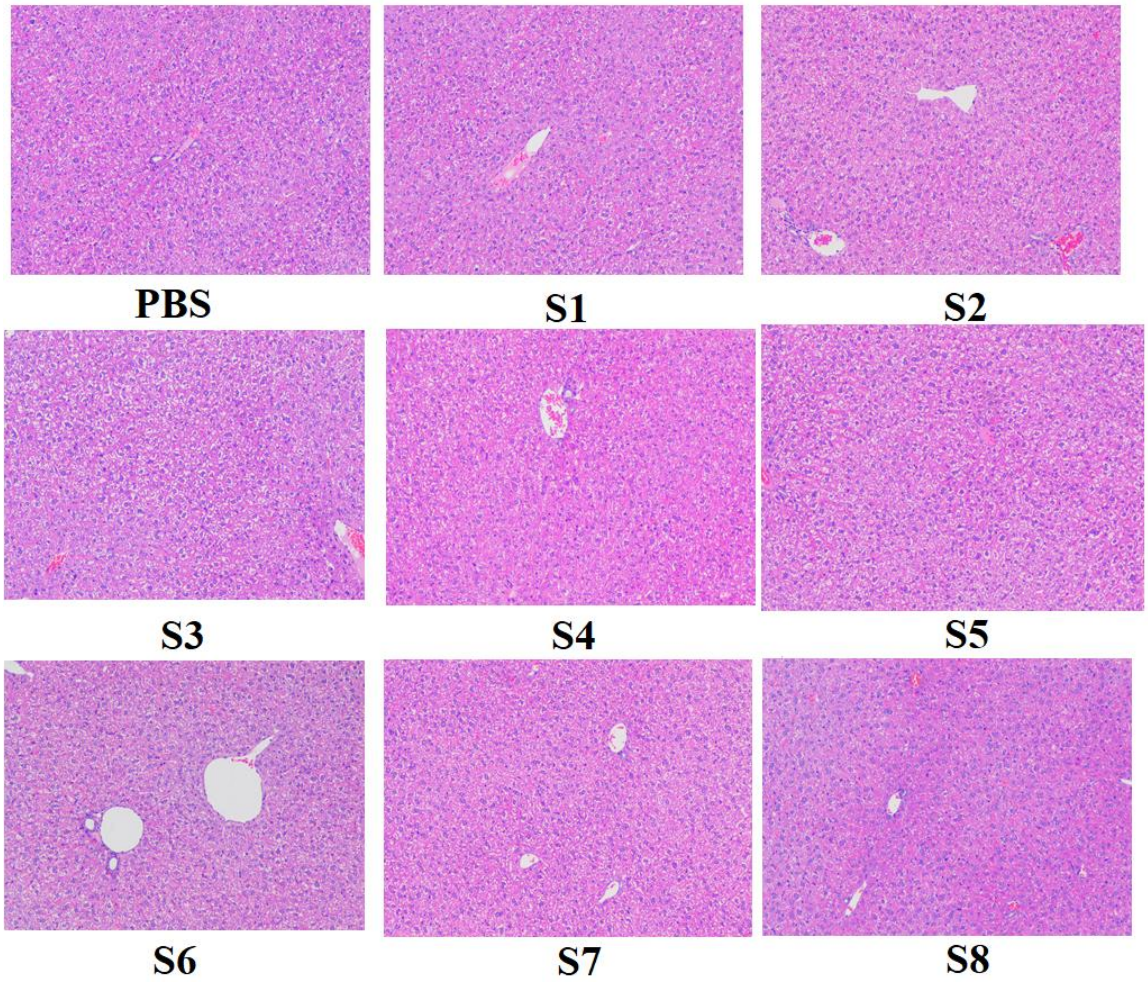


Figure S14. Hematoxylin and eosin (HE) stain of liver. The mice was treated with S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@ nanovehicle at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.

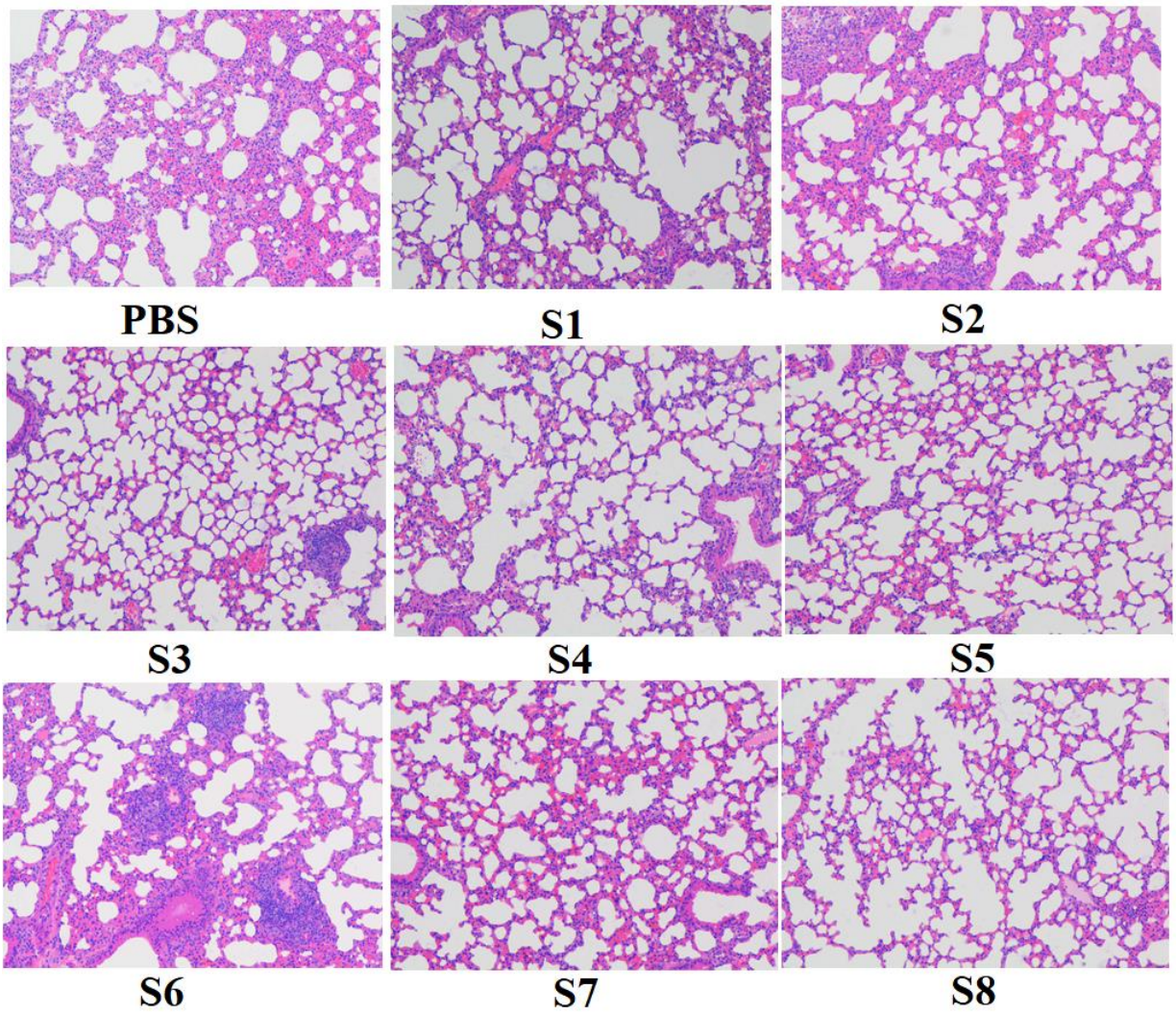


Figure S15. Hematoxylin and eosin (HE) stain of lung. The mice was treated with S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@ nanovehicle at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.

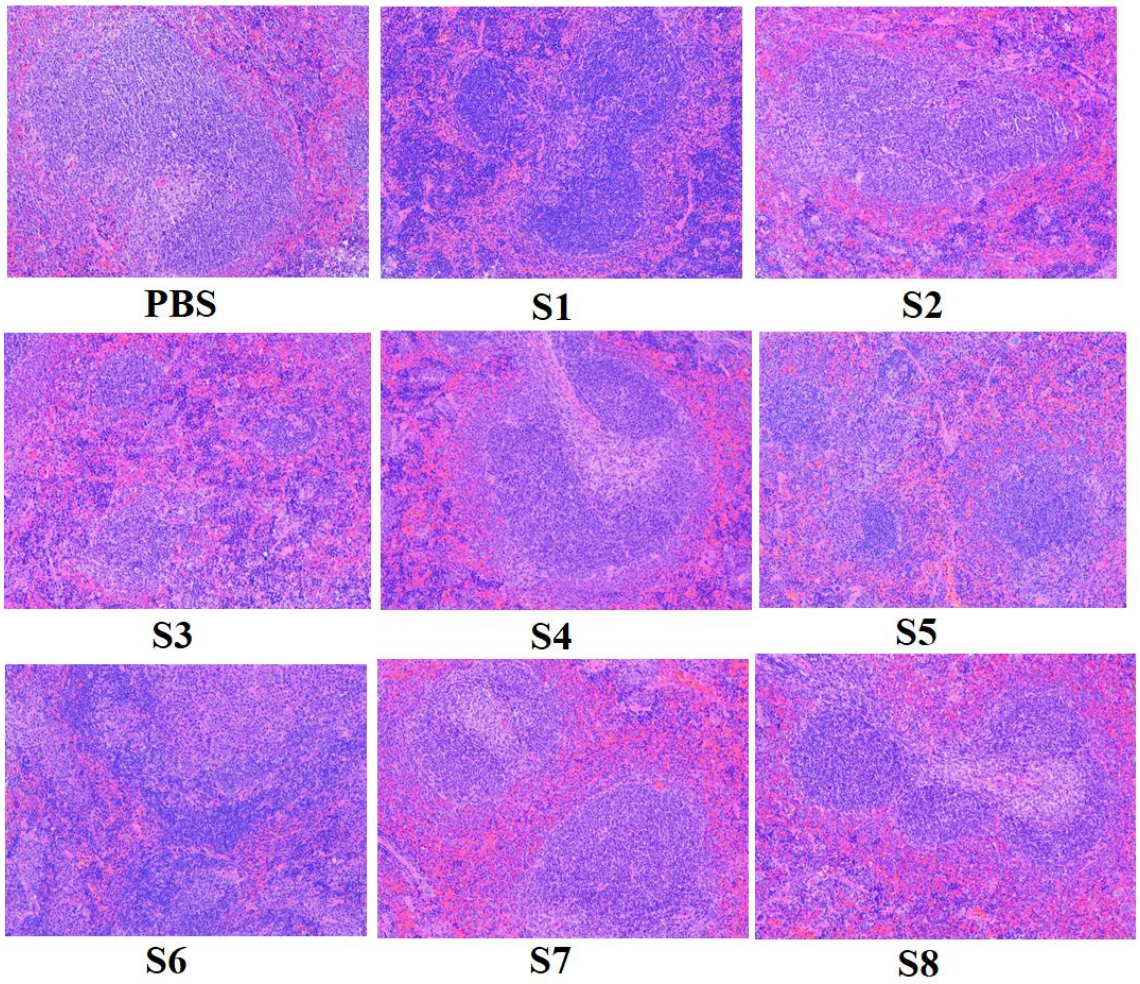


Figure S16. Hematoxylin and eosin (HE) stain of spleen. The mice was treated with S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@ nanovehicle total at dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.

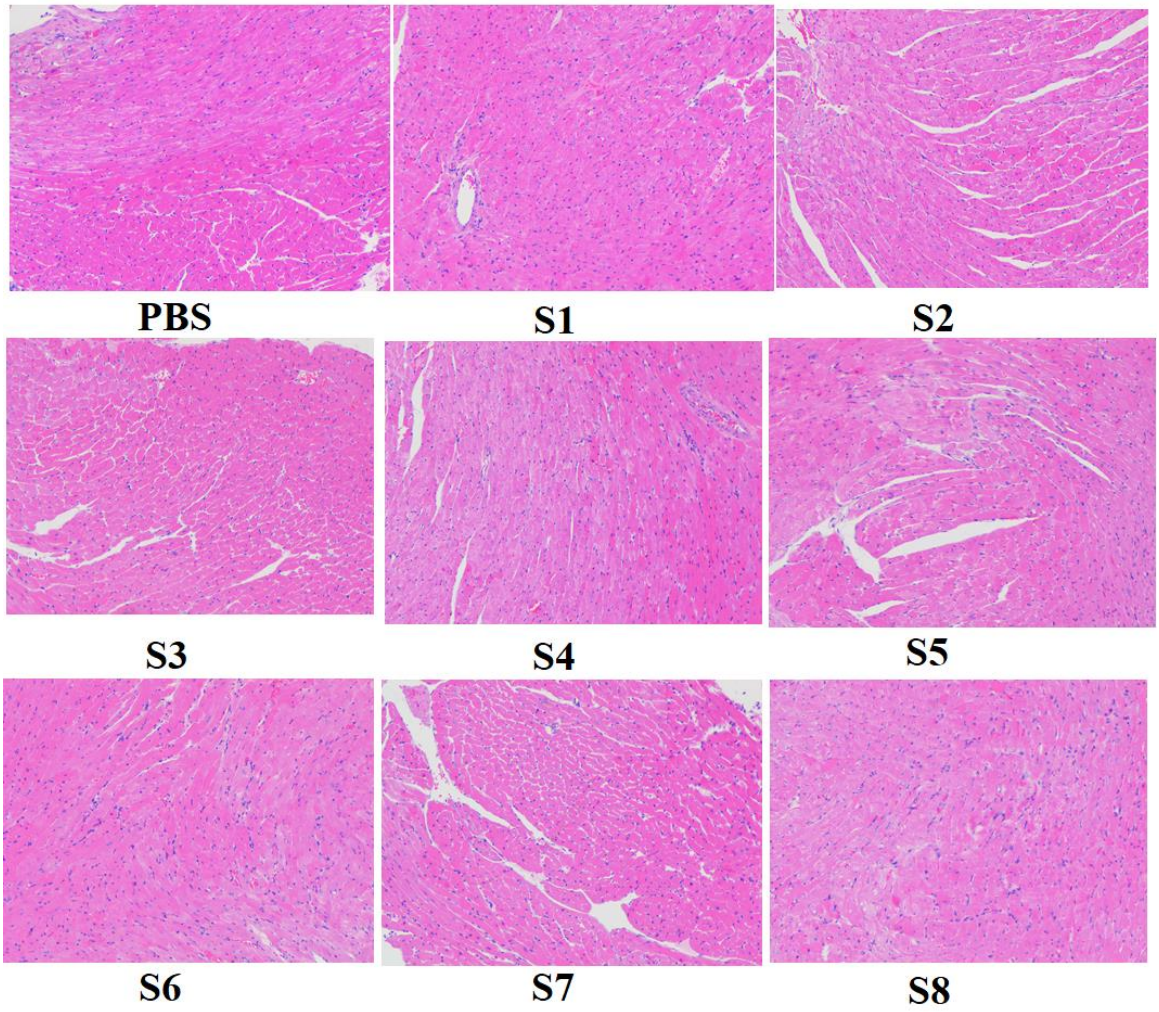
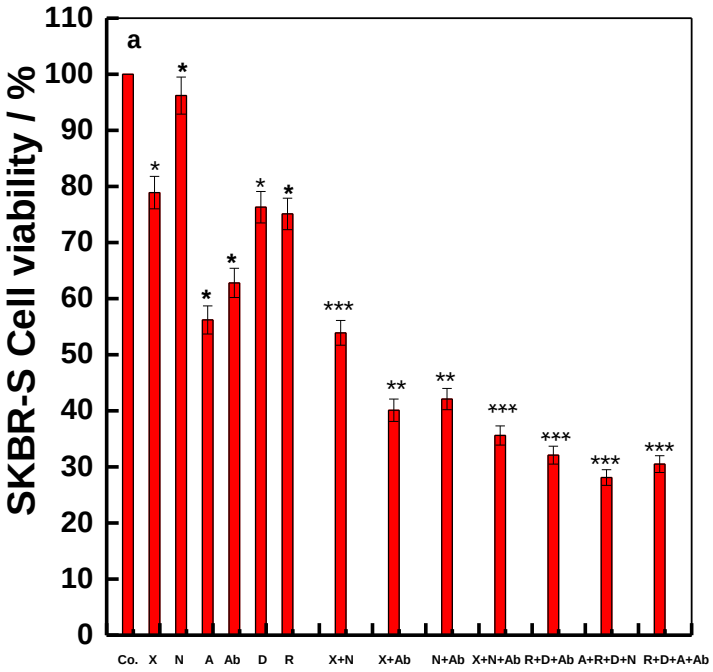


Figure S17. Hematoxylin and eosin (HE) stain of heart. The mice was treated with S1 (blank nanovehicle), S2 (Docetaxel@nanovehicle), S3 (Rapamycin@nanovehicle), S4 (Afatinib-nanovehicle), S5 (Docetaxel+Afatinib@nanovehicle), S6 (Rapamycin+ Afatinib@nanovehicle), S7 (Docetaxel+Rapamycin+Afatinib@nanovehicle), S8 (Docetaxel+ Rapamycin +Afatinib@nanovehicle at total dosage of 2.5mg/kg). The total dosage from S2 to S7 is 5mg/kg. The drug ratio for double and triple combinations is 1 to 1.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SKBR-S Cell viability / %
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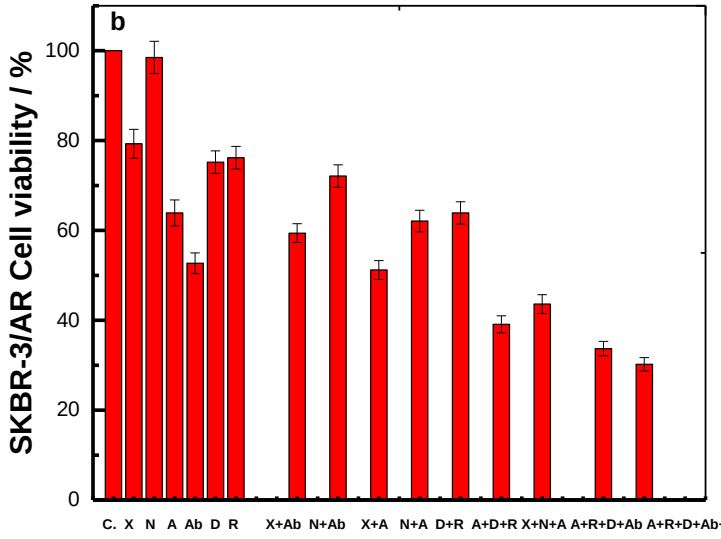
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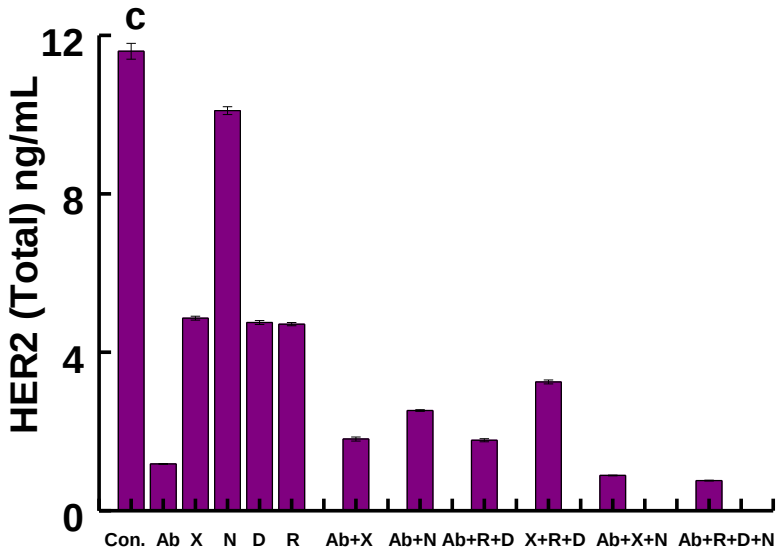

Figure S18. The synergism, MDR and HER2 study.

(a) Synergistic cell killing effect by drugs combination, anti-HER2 antibody and DNA drugs combination on SKBR-3 after 24 h incubation (Afatinib (A), DNA (N), DOX(X), anti-HER2 antibody (Ab), Rapamycin (R), Docetaxel (D). $C_X/C_{Afatinib}$ = 2:1; the other drug ratio is kept at 1:1; the total concentration of the single drug and multiple drugs is kept at 200 ng/mL ; DNA 10 nM; and multifunctional hybrid vesicles co-loaded with 10 μg/mL nano-magnetite, 10 nM folate conjugated gold nanorods and 400 μg/mL cNPs set up as control) using live/dead assay. The data were analyzed by a Student's *t*-test using the online version of Graph Prism software with the significance probabilities of $***p < 0.001$.

(b) The effect on Afatinib-resistant SKBR-3/AR HER2 positive breast cancer cells proliferation of Afatinib (A), DNA (N), DOX(X), anti-HER2 antibody (Ab), Rapamycin (R), Docetaxel (D). The single and total drug concentration is 200 ng/mL; C_D/C_A =1:1; C_D/C_R =1:1; C_R/C_A =1:1; $C_A/C_D/C_R$ = 1:1:1; DNA is 10 nM; vehicle with 400 μg/mL cNPs set up as control). All data represent mean ± S.D. (n = 3).

(c)The Human HER2 (Total) ELISA study for measurement of HER2 protein at optical density 450 nm on SKBR-3 cells. Afatinib (A), DNA (N), DOX(X), anti-HER2 antibody (Ab), Rapamycin (R), Docetaxel (D). The total drug concentration is 9 μg/mL; C_D/C_A =1:1; C_D/C_R

=1:1; C_R/C_A =1:1; $C_A/C_D/C_R$ = 1:1:1; DNA is 10 nM; vehicle with 400 μg/mL cNPs set up as control). All data represent mean ± S.D. (n = 3).

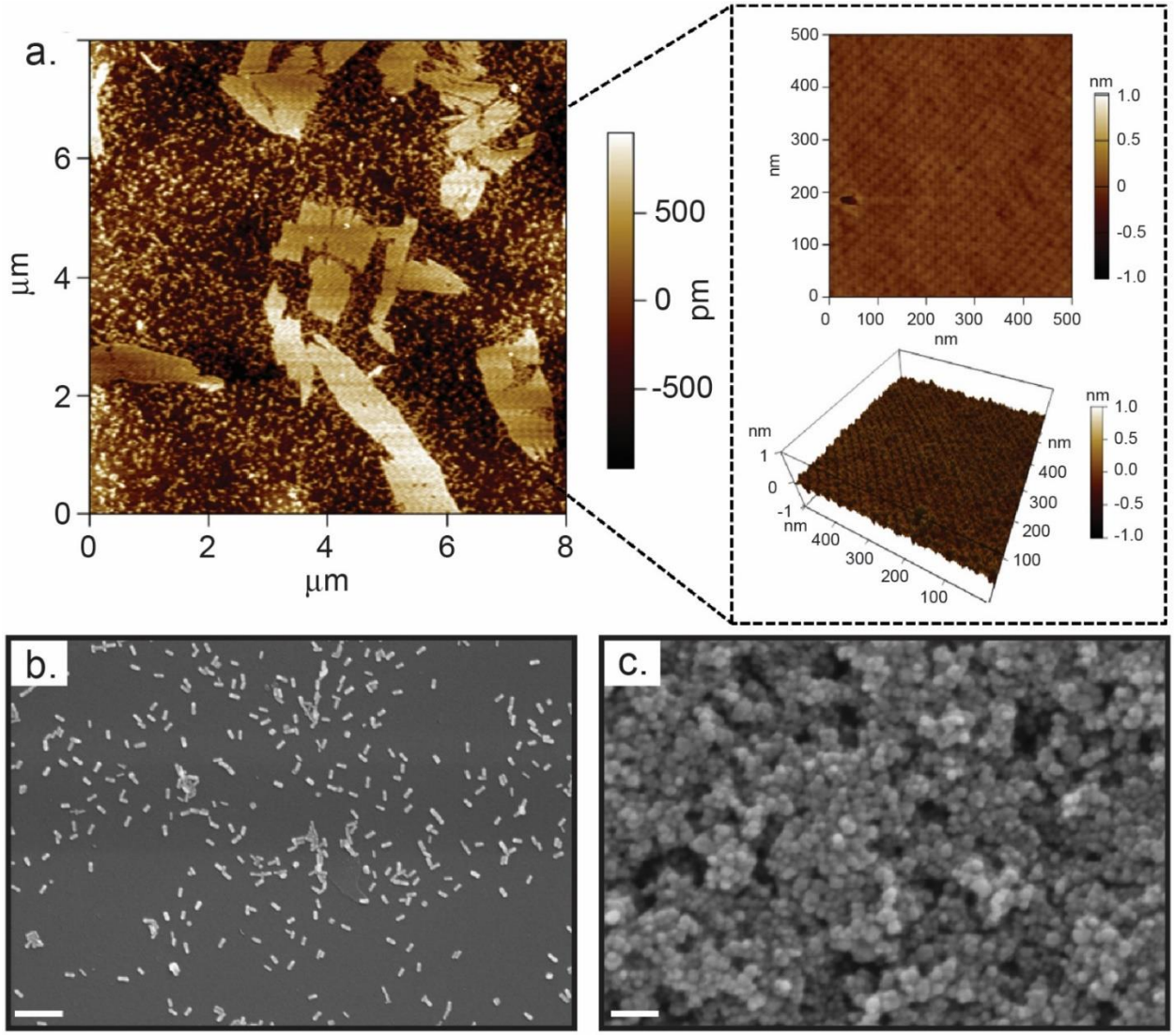

Figure S19. The morphology of dsDNA, gold nanorods and nano magnetite.

(a)AFM images of the synthesized SST AB DNA structures in air. (b) SEM image of gold nanorods (AuNRs); the scale bar denotes 200 nm; (c) SEM image of the nano magnetite; the scale bar denotes 50 nm.

Supporting Table

Table S1. The stability study of different hybrid polymersomes at different ratios of copolymer and phospholipid in the absence of cell using microscopy

Ratio between copolymer and phospholipid	Original polymersomes number	Polymersomes remaining number after one month
10:0	30	30
10:1	30	27
10:2	30	25
10:3	30	20

Supporting video

S1: The generation of w/o/w emulsion droplets templates.

S2: The magnetic responsiveness of the double emulsion templates.

References

1.Kong F, et al. (2015) Inhibition of Multidrug Resistance of Cancer Cells by Co-Delivery of DNA Nanostructures and Drugs Using Porous Silicon Nanoparticles@Giant Liposomes. *Advanced Functional Materials* 25:3330-3340.

2.Kong F, et al. (2016) Gold Nanorods, DNA Origami, and Porous Silicon Nanoparticle-functionalized Biocompatible Double Emulsion for Versatile Targeted Therapeutics and Antibody Combination Therapy. *Advanced Materials* 28:10195-10203.

3.Winfree E, Liu FR, Wenzler LA, Seeman NC (1998) Design and self-assenbly of two-dimensional DNA crystals. *Nature* 394: 539-544.