Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary Information

pH- sensitive nanodiamond co-delivery of Retinal and Doxorubicin boost breast cancer chemotherapy

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Experimental section

1 Cell culture

The MCF-7 cells were cultured in 7 mL DMEM medium enriched with a 1% penicillin−streptomycin solution and 10% FBS under 37°C in a 5% CO_2 humidified

atmosphere in T25 cell culture flask. The cells were passaged every 48 hours using 1 mL trypsin containing EDTA. MCF-7/ADR cells were cultured in 1640 medium containing 15% fetal bovine serum and 1% penicillin-streptomycin, the culture environment is the same as MCF-7 cells.

2 Drug loading

The UV-visible absorption spectrum of ASS shows peak at 330 nm. Serial dilutions of the drug were obtained, the spectra collected, and a standard curve was established with excellent linearity over the relevant range. ASS in the supernatant can be calculated. DOX has a characteristic absorption peak at 480 nm. Using $\mathrm{DOX}\varepsilon_{480\mathrm{nm}}=11500\ \mathrm{cm}^{-1}\ \mathrm{mol}^{-1}\ \mathrm{L}^{-1}$, the amount of DOX in the supernatant can be calculated. Drug loading is the initial amount of drug added minus the amount of supernatant.

3 High resolution mass spectrometry (HRMS) characterization of ASS

Take part of the ASS prepared, freeze-dry to remove the solvent, and then add chromatography-grade methanol to prepare a solution with a concentration of 1×10^{-6} g mL^{-1}, filter it with a 0.22 μm membrane, and then detection by high resolution mass spectrometry (HRMS).

4 In vitro stability of nanodrugs

The particulars of ND, NP, NPA and NPA@D were ultrasonically dispersed in a buffer solution of PBS (pH 7.4, 4 mL), respectively. After a while, it was taken photos under a daylight lamp. Then the dried NPA@D was ultrasonically dispersed in a buffer solution of PBS (pH 7.4, 2 mL), and stored in a refrigerator at 4°C in the dark. After proscribed periods, the NPA@D solution was centrifuged and the supernatant (2 mL) was taken out; the

supernatant was replaced with fresh PBS. Repeat the above steps until 120 days. The amount was measured in the supernatant by UV-visible spectrophotometer. The drug leakage rates were calculated using equation (1) to further investigate the stability of the NPA@D nanoparticles.

$$LR=\frac{\sum B_L}{C_T}\times 100\% \quad (1)$$

Among them, LR, B_L and C_T are the leakage rate, cumulative dissociation amount and respective loading amount of drugs.

5 pH sensitive in vitro

Take some of NPA particulars in 2 mL of sodium acetate buffer solution (ABS pH 4.5, containing Tween 80, w:v = 10%), ultrasonically dispersed for 30 min, and then placed on a magnetic stirrer at 37°C to stir for 3 h. After that, the supernatant was removed by centrifugation and freeze-dried. The dried product was dissolved and detected by a high-resolution mass spectrometer (HRMS).

6 Cell morphologic observation

For the morphological analysis induced by free DOX, the MCF-7 cells were seeded in 35 mm Petri dishes at a density of 2×10^5 cells per dish for 18 h, and treated with DOX (3 μg m L^{-1}) for 24 h, 48 h and 72 h, respectively. After the treatment, the cells were washed with PBS, and the cells were fixed with 4% paraformaldehyde (400 μL) for 8 min at room temperature. The morphologies were monitored via optical microscopy.

7 Cellular uptake and mechanism

The MCF-7 cells were inoculated in 10 mm cell culture dish at a density of 2.0 × 10^5/dish and allowed to attach overnight, the prepared culture medium containing NPA@D nanodrug (5 μg mL^{-1} of DOX equivalent) was added and incubated for 1 h, 3 h, 5 h, 7 h, 15 h, 20 h, 21 h, and 23 h, respectively. When the incubation was finished, the samples were washed with PBS (pH 7.4) and harvested by trypsinization, which were collected and then analyzed by flow cytometer.

To determine which the mechanism of NPA@D nanomedicine entry into tumor cells was. Adhered MCF-7 cells were pretreated with low temperature (4°C), NaN_3 (0.8 M), Sucrose (0.45 M), and M-β-CD (0.01 M) for 30 min, and then the prepared culture medium containing NPA@D (5 μg mL^{-1} of DOX equivalent) was added, and the cells in the control group were untreated. Before the flow cytometry testing, the cells were washed with PBS to remove NPA@D, then harvested and collected.

8 Transwell assay

Transwell assay was applied to test the vertical migration capability of MCF-7 cells. The cells (1×10^4 cells per well) were seeded in the upper chamber of inserts in 24-well plates with fresh serum-free media (8 μm pore size, Corning Incorporated), and ATR (0.75 μg mL^{-1}), DOX (3 μg mL^{-1}) NPA (0.75 μg mL^{-1} of ATR equivalent), NP@D (3 μg mL^{-1} of DOX equivalent) and NPA@D (3 μg mL^{-1} of DOX equivalent, 0.75 μg mL^{-1} of ATR equivalent) suspending were placed in the upper compartment. The MCF-7 cells exposed to fresh serum-free media were used as control group. After incubating at 37°C in a 5% CO_2-humidified incubator for 36 h and 48 h, unmigrated cells on the filter

membrane were removed using a cotton swab, whereas the cells that migrated to the lower membrane surface were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet and viewed by optical microscope. Number of cells was performed by ImageJ software.

9 Scratch assay

MCF-7 cells were seeded in six-well plates (density: 1 × 10^5 cells/well) to form a confluent monolayer of MCF-7 cells, then, a tiny pipet tips were used to form a cross wound by manual scraping, the cells were washed with PBS (pH 7.4) to move away cell debris before adding different nanodrugs (3 μg mL^{-1} of DOX equivalent). The wounds were observed under a Nikon microscope at 0, 36 and 48 h, and marked the width of the scratch on the picture. The wound closure rate (WC) and migration inhibition rate (MIR) was calculated by using the following formula.

$$WC=(1-\frac{SW_t}{SW_0}) \times 100\% \quad (2)$$

$$MIR=(1-\frac{WC_{treatment}}{WC_{control}})\times 100\% \quad (3)$$

where SW_t is the scratch width at 36 h or 48 h, and SW_0 is the initial scratch width. $WC_{treament}$ represents the cell scratch healing rate of the drug group, and $WC_{control}$ represents the scratch healing rate of the control.

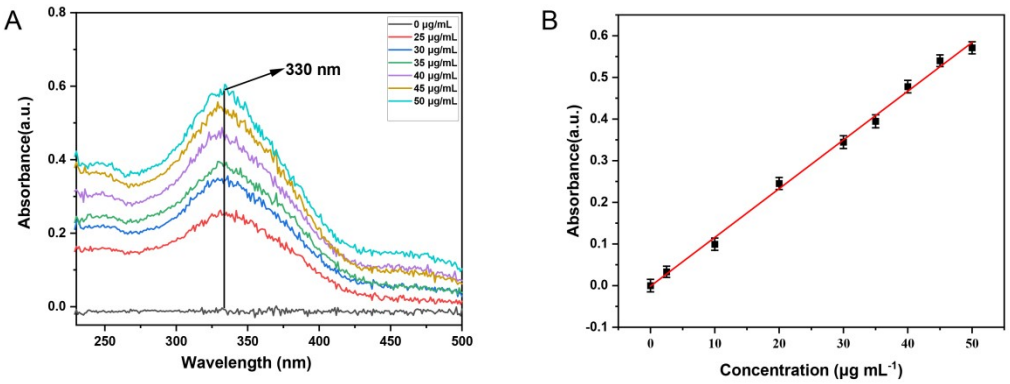
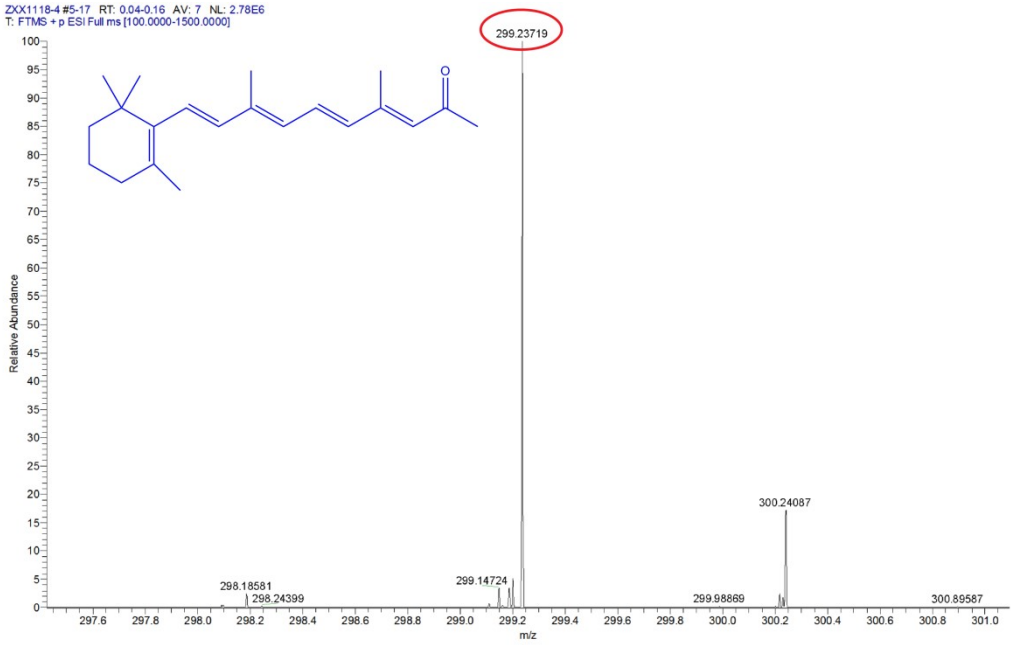
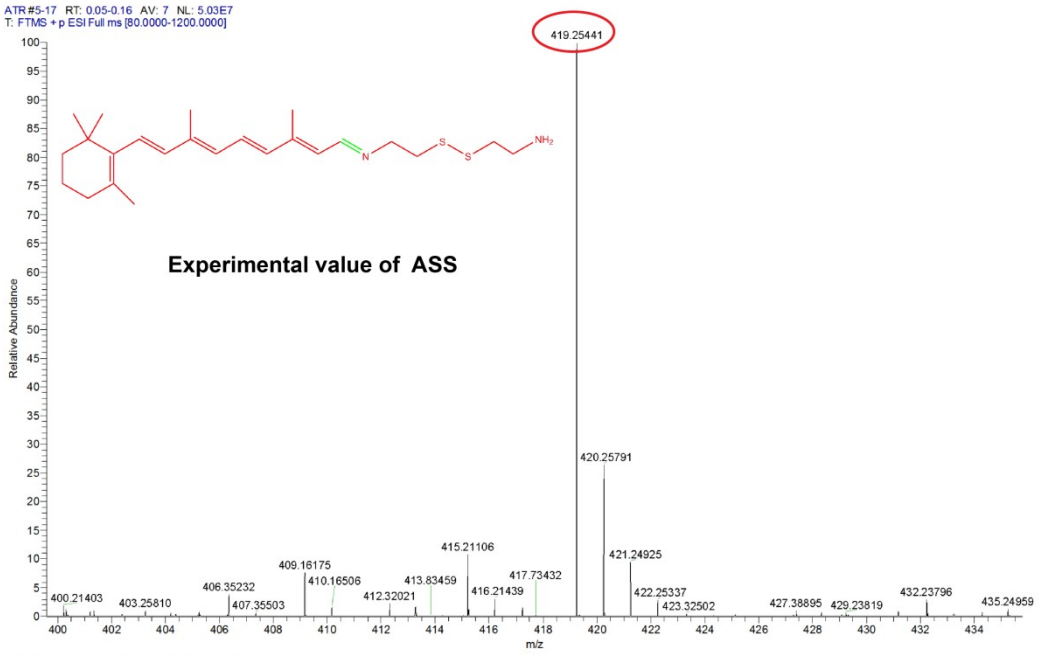


Figure S1 (A) UV-Vis absorption spectrum of ASS. **(B)** Standard curve.

A


ZXX1118-4 #5-17 RT: 0.04-0.16 AV: 7 NL: 2.78E6
T: FTMS + p ESI Full ms [100.0000-1500.0000]
299.23719
300.24087
298.18581
298.24399
299.14724
299.98869
300.89587
Relative Abundance
m/z

B


ATR #5-17 RT: 0.05-0.16 AV: 7 NL: 5.03E7
T: FTMS + p ESI Full ms [80.0000-1200.0000]
419.25441
Experimental value of ASS
420.25791
421.24925
415.21106
409.16175
410.16506
406.35232
413.83459
417.73432
412.32021
416.21439
400.21403
403.25810
407.35503
422.25337
423.32502
427.38895
429.23819
432.23796
435.24959
Relative Abundance
m/z

C

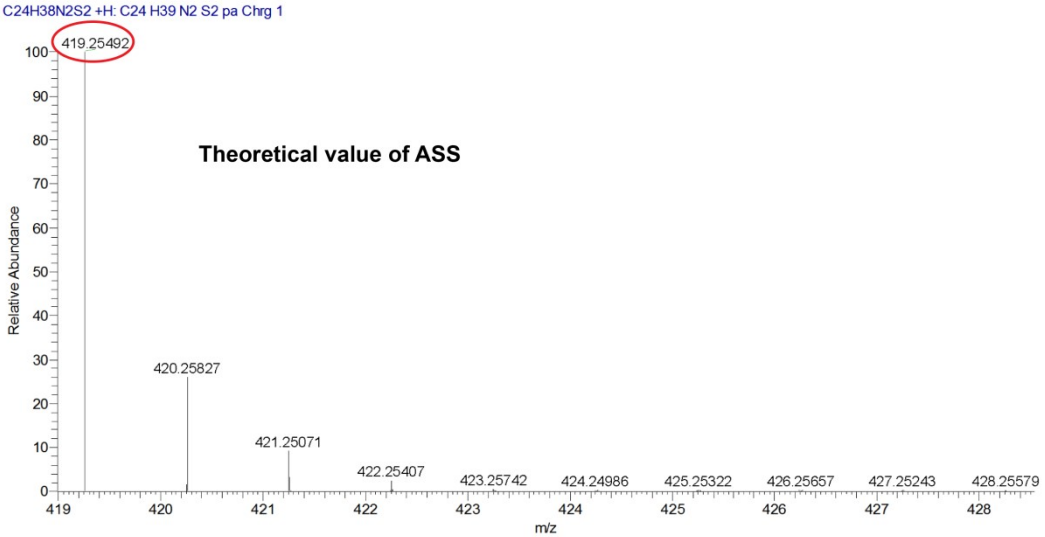

C24H38N2S2 +H: C24 H39 N2 S2 pa Chrg 1
419.25492
Theoretical value of ASS
420.25827
421.25071
422.25407
423.25742
424.24986
425.25322
426.25657
427.25243
428.25579
Relative Abundance
m/z

Figure S2 HRMS of **(A)** ATR; **(B)** Experimental value of ASS; **(C)** Theoretical value of ASS. HRMS (ESI, m/z): $[M+H]^+$Calcd for $C_{24}H_{38}N_2S_2$: 419.25492, found 419.25441.

Table. S1 Particle size, Zeta potential and polydispersity index of different nanoparticles

	Diameter(nm)	Zeta potentials(mV)	PDI
ND	170.0±1.5	-20.3±1.0	0.198
NP	193.5±3.5	-21.6±0.8	0.137
NPA	225.0±4.3	-23.2±0.5	0.122
NPA@D	272.6±2.7	-19.9±1.4	0.109

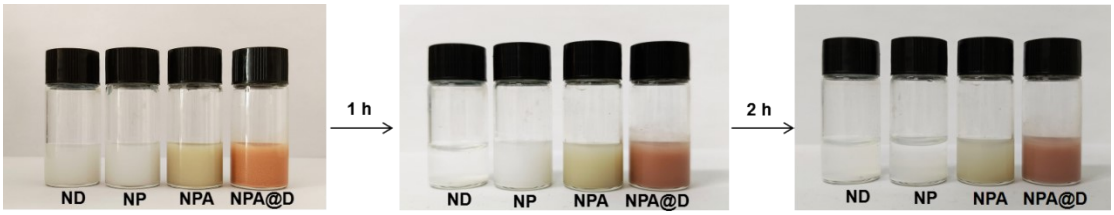


Figure S3 The dispersibility of different nanoparticles (1 mg mL^{-1}) in PBS (pH 7.4)

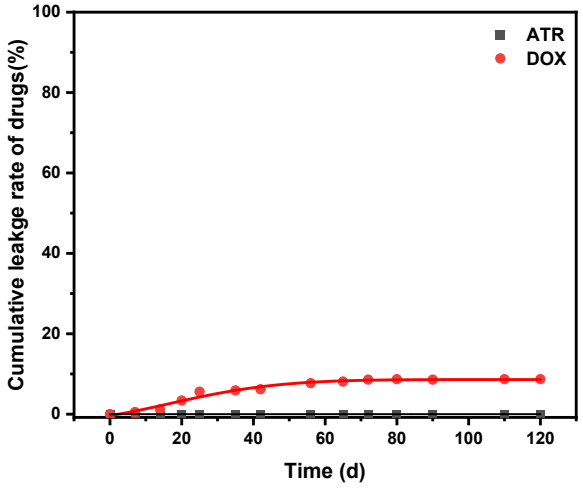
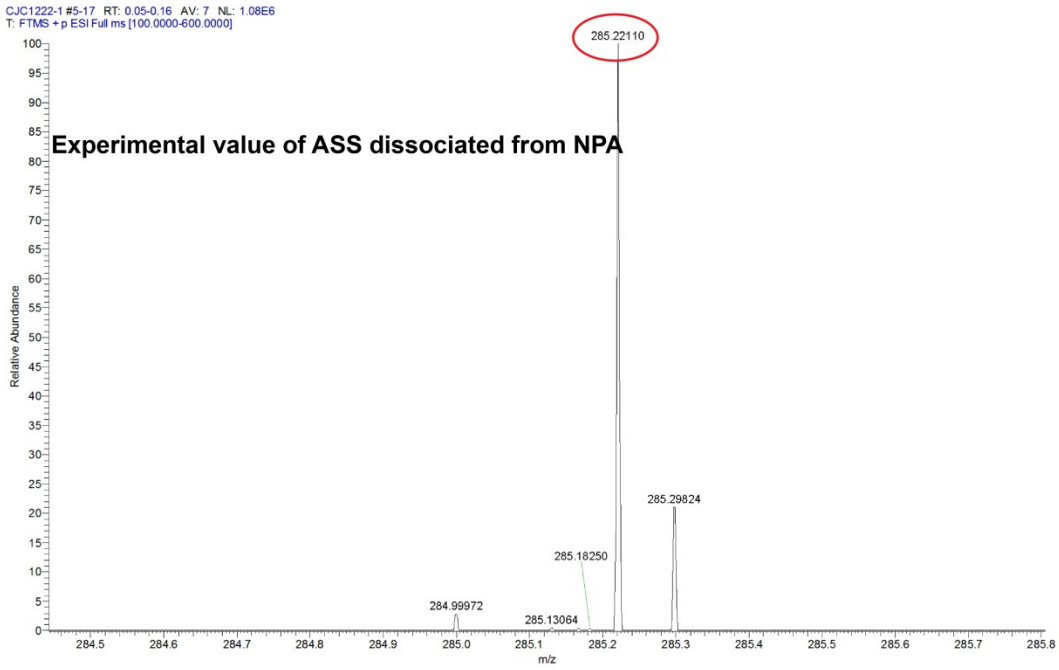
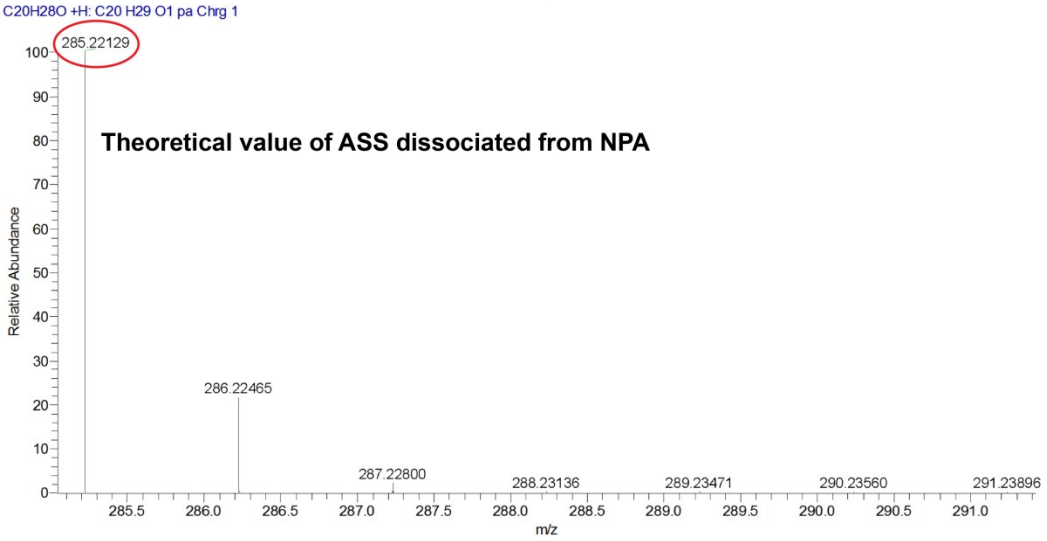


Figure S4 The stability of NPA@D (1 mg mL^{-1}) in the PBS stored at 4ºC and pH 7.4

A


CJC1222-1 #5-17 RT: 0.05-0.16 AV: 7 NL: 1.08E6
T: FTMS + p ESI Full ms [100.0000-600.0000]
285.22110
Experimental value of ASS dissociated from NPA
285.29824
285.18250
284.99972
285.13064
Relative Abundance
m/z

B


C20H28O +H: C20 H29 O1 pa Chrg 1
285.22129
Theoretical value of ASS dissociated from NPA
286.22465
287.22800
288.23136
289.23471
290.23560
291.23896
Relative Abundance
m/z

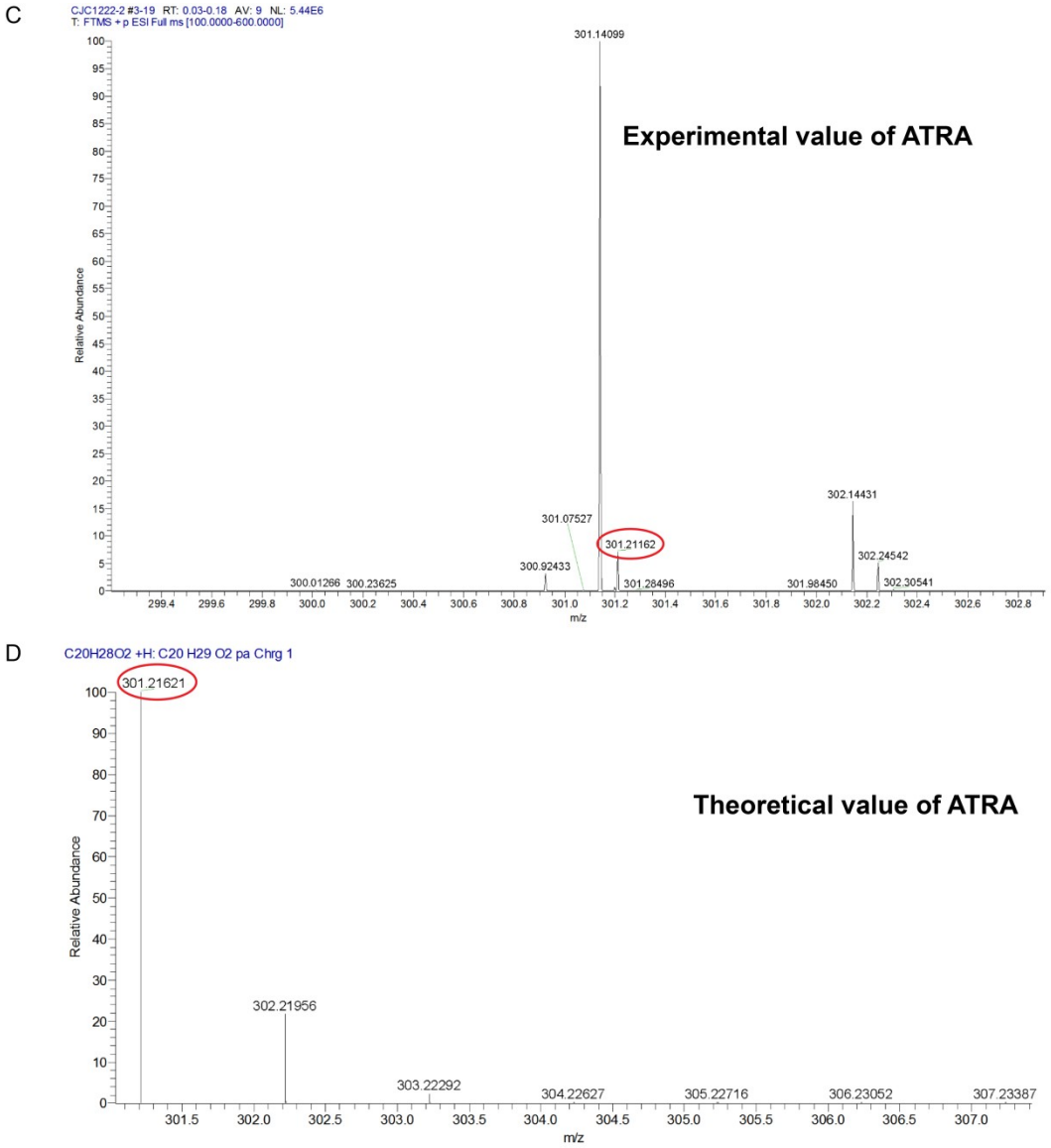


Figure S5 HRMS of ATR dissociated from NPA and ATRA in acetic acid buffer solution (ABS pH 4.5). **(A)** Experimental value of ASS dissociated from NPA. **(B)** Theoretical value of ASS dissociated from NPA. HRMS (ESI, m/z):[M+H]$^+$Calcd for $C_{20}H_{28}O$: 285.22129, found 285.22110. **(C)** Experimental value of ATRA in ABS. **(D)** Experimental value of ATRA. HRMS (ESI, m/z):[M+H]$^+$Calcd for $C_{20}H_{28}O_2$: 301.21621, found 301.21162.

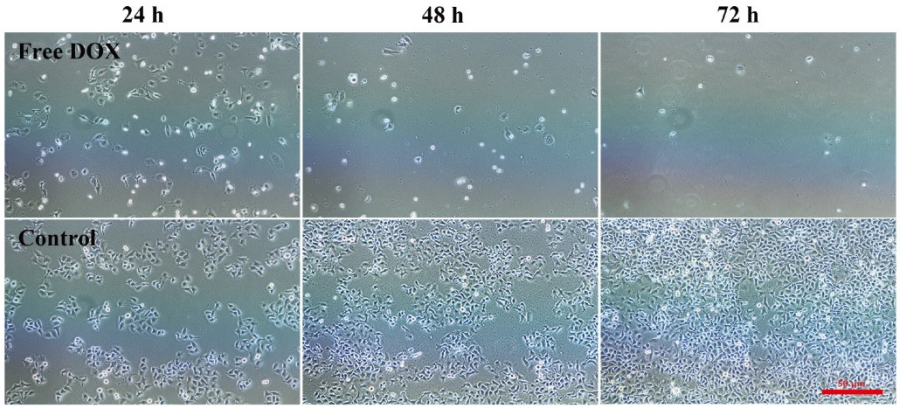


Figure S6 The effect of MCF-7 cell morphology with free DOX (3 μg m L^{-1}) for 24 h, 48 h and 72 h (for each group, n = 3), (scale bars: 50 μm).

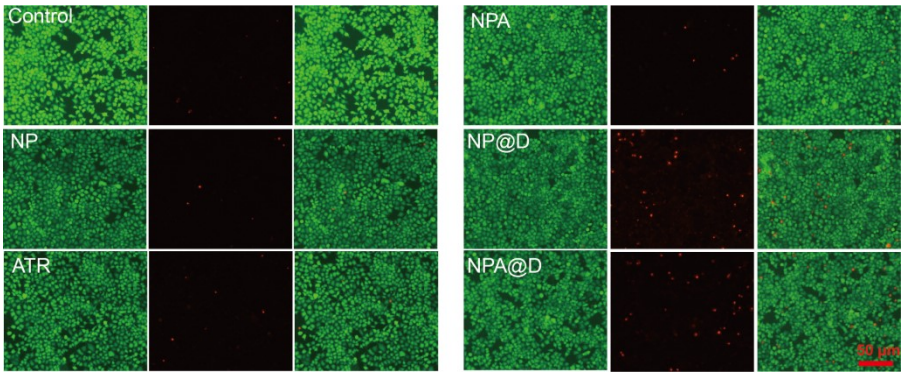


Figure S7 Calcein AM/PI staining to visualize HL-7702 cells viability treated with NP, ATR (0.75 μg mL^{-1}), DOX (3 μg mL^{-1}), NPA (ATR 0.75 μg mL^{-1} equivalent), NP@D (DOX 3 μg mL^{-1} equivalent), and NPA@D (DOX 3 μg mL^{-1} equivalent, ATR 0.75 μg mL^{-1}) for 48 h (for each group, n = 3), (scale bar = 50 μm).

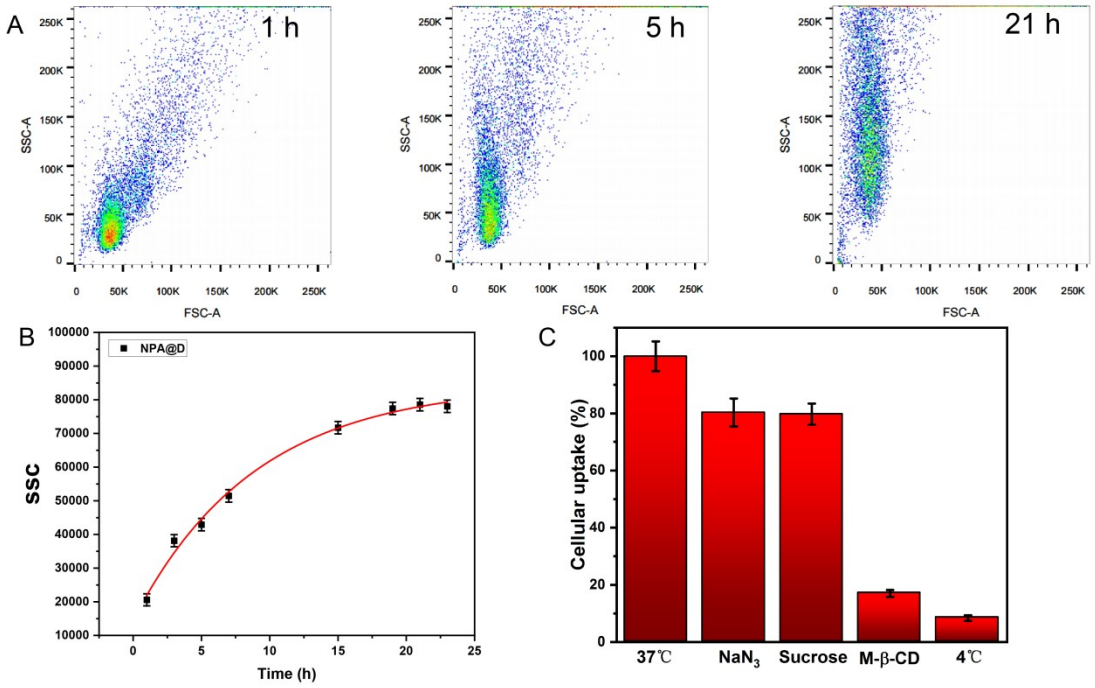


Figure S8 Cells internalize NAP@D nanoparticles. **(A)** The variation of side scatter (SSC) quantified from a minimum of 10,000 cells by CellQuest software after MCF-7 cells were treated with NAP@D (5 μg mL^{-1} of DOX equivalent) for different incubation periods. Y-axis is Side Scatter (SSC-A), which indicated intracellular particle's complexity. X-axis is forward scatter (FSC-A) of the cells and represents the size of the cells. **(B)** The kinetics of the cellular uptake of NPA@D by MCF-7 cells. **(C)** Cellular uptake of NAP@D by MCF-7 cells under different treatment conditions. The results were obtained from three separate experiments and the bar represents mean ± standard error.

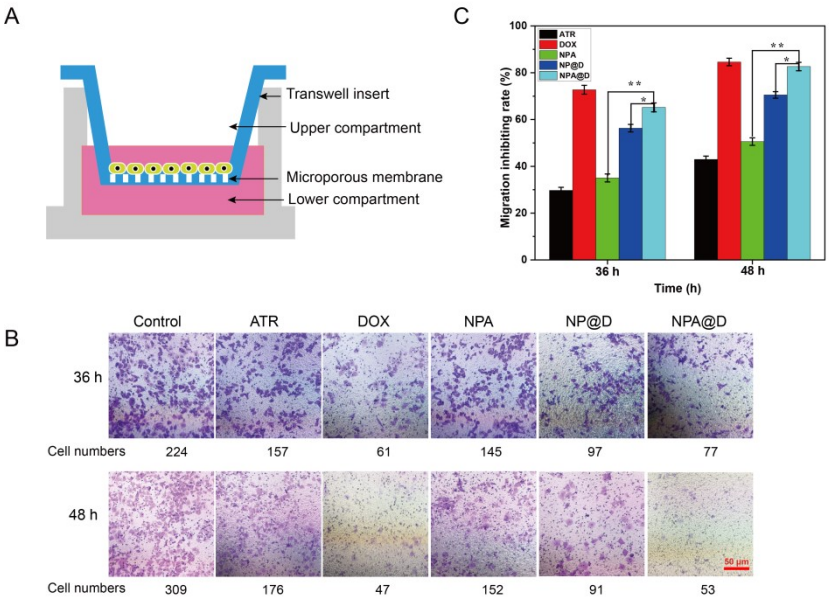


Figure S9 Cell migration analysis. **(A)** Transwell migration cell model. **(B)** Representative photographs of the transwell migration cell treated with ATR (0.75 μg mL^{-1}), DOX (3 μg mL^{-1},) NPA (ATR 0.75 μg mL^{-1} equivalent), NP@D (DOX 3 μg mL^{-1} equivalent) and NPA@D (DOX 3 μg mL^{-1} equivalent, ATR 0.75 μg mL^{-1} equivalent) after 36 h and 48 h, and the number of cells in each photograph are attached below (for each group, n = 3), (scale bar = 50 μm). **(C)** Effects on the migration inhibition rate for MCF-7 cells - treated by various drugs.

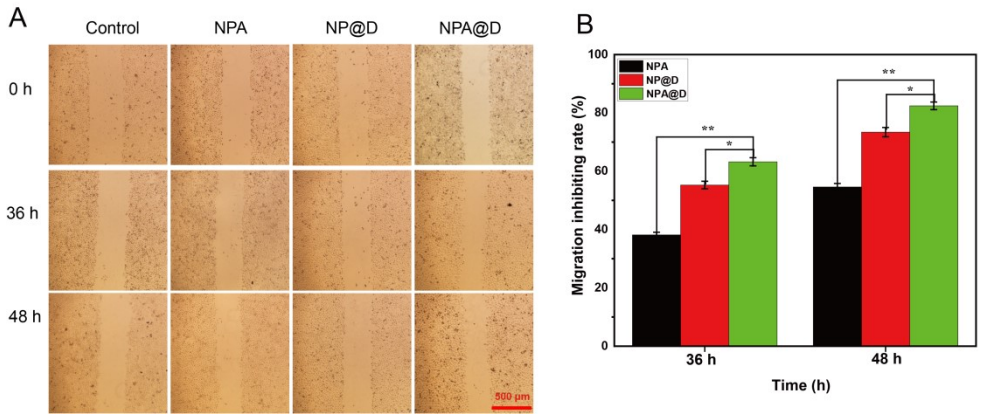


Figure S10 Cell migration. **(A)** Cell scratch pattern after co-incubation with different nanoparticles in 36 h and 48 h (for each group, n = 3), (scale bar = 500 μm). **(B)** Inhibition rate of cell migration.