Electronic supplementary information

Beyond chemotherapeutics: Cisplatin as a temporary buckle to fabricate drug-loaded nanogel

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Materials

Cisplatin, Rhodamine B (Rb), o-phenylenediamine (OPDA) and Dimethylformamide (DMF) were from Sigma-Aldrich (St. Louis, MO, USA). Dasatinib (Dasa) and Doxorubicin (Dox) were purchased from LC Laboratories (Boston, MA, USA). Hyaluronan (HA) (MW1000 kDa) was purchased from Lifecore Biomedical Co. (Chaska, MN, USA). LC-MS analyses were performed with Waters Acquity UPLC - Waters Zspray^{TM} (Milford, MA, USA).

Preparation of the nanogel (NG)

Cisplatin (15 mg/ml) in water was preheated at 90 °C to allow the cisplatin dissolved. Rb, Dox were dissolved in water and Dasa was dissolved in DMF at the concentration of 10 mg/ml. Hyaluronan (5 mg/ml) was dissolved in water. First, 150 µl cisplatin was mixed with 100 µl Rb or 60 µl Dasa or 50 µl Dox on 90 °C. Appropriate volume of water was added to fix the volume at 400 µl. Then 800 µl of HA was mixed with above drug solution and the mixture was incubated on 90 °C for predetermined hours. The heating time was as follow: 3 hours for Rb, 2.5 hours for Dasa and 2 hours for Dox. After the heating, the mixture was cooled on ice for 15 minutes and subjected to dialysis (MWCO= 3.5 kDa) in 1L of water at room temperature or PBS at 37 °C. For those mixture dialyzed in water, the products were designated as HA/Cis/Rb, HA/Cis/Dasa and HA/Cis/Dox and the mixtures dialyzed in PBS were named as HA/Rb, HA/Dasa and HA/Dox since cisplatin would be removed with the existence of chloride in PBS. Similar to above procedure, HA was also mixed with only Cis or Rb with a heating time of 3 hours followed by dialysis in water. The corresponding products were referred to HA/Cis and

HA@Rb, respectively. The dialysis was conducted for 3 days with water or PBS changed regularly. During dialysis the water or PBS was collected to quantify the drug unbound to the NG. The quantification of Rb and Dasa was dependent on their absorbance peak (544 nm and 325 nm for Rb and Dasa, respectively) according to the corresponding standard curve. The quantification of Dox was done using the Dox fluorescence with excitation and emission set at 490 nm and 590 nm, respectively. The acquired fluorescent intensity was translated to Dox content using the free Dox as standard curve. To measure the cisplatin, a modified OPDA method was used as described below. Drug loading efficiency was expressed as follow:

$$Drug\ loading\ efficiency = \frac{Weight\ of\ drug\ in\ NG}{Total\ weight\ of\ NG} \times 100\%$$

OPDA method

The OPDA method to quantify free cisplatin was relied on the reaction product of OPDA-cisplatin [1, 2]. In order to quantify cisplatin in NG, the OPDA method was modified by changing the reaction medium to 9% NaCl solution in which the ciaplatin would be released due to reversed co-ordination. Briefly, the NG was diluted at the rate of 100 folds using 9% saline and then 250 µl of diluted NG was mixed with equal volume of 1.2 mg/ml OPDA in DMF. The mixture was heated on 100 °C for 10 min. After that, 200 µl of reaction product was subjected to absorbance measurement at 705 nm. To verify the detecting efficacy of modified OPDA method, the reaction conducted in water and 9% NaCl was compared. A 250 µl of 0.08-20 µg/ml cisplatin in water or in 9% NaCl was reacted with equal volume of OPDA in DMF for 10 min at 100 °C to acquire the cisplatin standard curve using water and saline as reaction medium. The NaCl inhibited the reaction but improved the linearity of the standard curve as shown in Figure S4b. After

that NG of HA/Cis and HA/Cis/Rb with predetermined cisplatin concentration was used to confirm the accuracy of the modified OPDA method. It turned out the reaction in saline could measure almost all the cisplatin in NG while reaction in water only detected around 85% of the cisplatin (Figure S4c). The existence of HA, Rb, Dasa and Dox didn't interfere with the absorbance of OPDA-cisplatin product since their absorption was far away from 705 nm. In the case of Rb-involved NG, the absorption spectra from 300-800 nm were recorded after the reaction with OPDA.

Dasa and Dox integrity examination

In order to verify the integrity of Dasa and Dox after heating treatment during the drug loading procedure, LC-MS analyses were performed with Dasa and Dox before and after heating. Dasa (1 mg in 1 mL H_2O/CH_3CN (1:1) solution) and Dox (1 mg in 1 mL H_2O) were heated at 90 °C for 2.5 hours and 2 hours, respectively. The solution of Dasa and Dox before and after heating were subjected to LC-MS and the results showed the integrity of both Dasa and Dox with the same retention time and mass spectrum (Fig S13, a-d).

For cytotoxic analysis of unheated and heated Dasa or Dox, Dasa in DMSO and Dox in water heated with the same condition used above. Around 4000 MDA-MB-231 cells in 96 wells plate were incubated with 0, 0.1, 1, 10, 100, 1000, 10000, 20000 nM unheated or heated Dasa and 0, 1, 10, 50, 100, 500, 1000, 10000 nM Dox or heated Dox for 3 days. Then the cell viability was evaluated using MTS assay described as below. Each drug treatment was conducted at least in triplicate.

Dynamic light scattering (DLS) analysis

For size measurement, 100 µl of NG was diluted using 1 ml water and the diluted solution was analyzed using a Zetasizer Nano-S machine (Malvern Instruments, UK). Each sample was measured in triplicate. For zeta potential, 150 µl of NG was mixed with 1 ml water. The measurement was conducted on a Zeta PALS analyzer (Brookhaven Instruments, Holtsville, NY).

UV-Vis-NIR absorption and fluorescent spectra

The NG was diluted using water with HA set at 0.1 mg/ml concentration. The absorption from 200-800 nm was recorded using Cary 60 UV-Vis Spectrophotometer. The fluorescent spectra of Rb and Dox-containing NG were determined using Tecan Infinite M1000 Pro Microplate Reader. The concentration of Rb in NG together with free Rb was fixed at 10 µg/ml while Dox concentration was set at 40 µg/ml. For Rb fluorescent reading, the excitation was 540 nm and emission was from 560-710 nm. For Dox fluorescent spectra, the excitation was 490 nm and emission was from 520-720 nm.

Transmission electron microscopic (TEM) observation

A 20 µl of NG was first diluted with 30 µl of water (1.3 mg/ml in HA). Then 5 µl of diluted NG was dropped on the grid. After 2 minutes, the grid was washed using 4 drops of 1.5% Uranyl acetate and then stained in one drop of uranyl acetate for 1 min. After that, the staining solution was dried using the paper. The morphology of NG was observed using a JEOL 1400 Transmission Electron Microscope with different

magnifications. At least 200 particles were counted from the TEM image to acquire the size distribution of the NGs.

Drug release of NGs

The PBS containing 5 mg/ml BSA, a concentration similar to the total concentration of serum proteins in commonly used cell culture medium, was used as drug release medium. The contact with BSA was used to simulate the interaction between serum proteins and NGs. Briefly, 1ml of NG was mixed with equal volume of PBS/BSA and sealed in dialysis tube (MWCO=3.5 kDa). The dialysis tube was immersed in 80 ml PBS and incubated at 37 °C with a rotation speed of 100 rpm. At the time points of 1h, 2h, 4h, 8h, 12h, 24h, 48h and 72h, 1.6 ml of PBS was collected and equal volume of fresh PBS was added in the beaker. The amount of released drug was quantified based on the specific absorbance according to the related standard curve.

NG degradability in the presence of HAase

To simulate the drug release of Rb in HA/Rb under the intracellular condition, hyaluronidase enzyme (HAase, #H3506, Sigma) was introduced in HA/Rb solution in PBS and incubated at 37 °C. The HAase concentration was set at 0, 100 and 500 U/ml and HA in HA/Rb was fixed at 167 μg/ml. At predetermined time point, the fluorescent intensity of Rb was monitored by Tecan Infinite M1000 Pro Microplate Reader with the excitation and emission set at 560 nm and 580 nm, respectively. To confirm the degradability of HA/Rb after the HAase treatment, HA/Rb was incubated with no HAase and 500 U/ml HAase for 2 days at 37 °C. Then the TEM observation was conducted as described above.

Cell culture

Human breast cancer cell line MDA-MB-231and MCF-7 cells and fibrosarcoma cell line HT-1080 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in DMEM medium containing 10% FBS and 100 U/ml penicillin and streptomycin using an incubator with humidified air containing 5% CO_2 at 37 °C.

Fluorescent microscopy

Around 4000 HT1080 cells were seeded in black 96 well with clear bottom and left overnight to allow cell adherence. Cells were incubated with HA/Cis/Rb and HA/Rb at the concentration of 83 μg/ml HA. Cell incubated in only medium was set as control. After 1 and 3 days of incubation, cells were imaged using EVOS fluorescent microscope (Life Technologies) in phase contrast and RFP channel.

To probe the intracellular localization of HA/Rb, HT-1080 cells in the chamber slide was incubated with HA/Rb containing 4 μg/ml Rb for 30 min. Then the cells were washed twice using PBS and followed by staining with 2 μM LysoTracker blue and 50 nM MitoTracker green in medium for 1 hour. Cells were washed twice with PBS and incubated in phenol red free medium for fluorescent microscopic observation. The images collected were designated as 1h time point. After 5 hours further incubation, the cells were imaged again under fluorescent microscope and the images were named as 6h time point. The DAPI, GFP and RFP channels were used to image the lysosome, mitochondria and Rb.

To probe the targeting potential of HA/Rb, MDA-MB-231 cells and MCF-7 cells in 96 well were incubated with HA/Rb of 4 μg/ml Rb and 33 μg/ml HA. For competition assay, the cells were incubated with same concentration of HA/Rb but with 0.25 mg/ml extra free HA. After 90 min incubation, cells were washed twice and imaged immediately in RFP channel (time point of 0h). After further 23 hours of incubation, cells were imaged again using the fluorescent microscope (time point of 24h).

CD44 expression

About 100 μl of MDA-MB-231 or MCF-7 cells (5x 10^4 cells) were incubated with 1 μl of APC-labeled anti-human CD44 antibody (Biolegend, San Diego, CA) for 30 minutes on ice. Then cells were rinsed twice using PBS to remove the unbound antibody. The fluorescence of the cells was analyzed using a Gallios flow cytometer (Beckman Coulter) in FL6 channel. Cells with no staining were set as negative control.

MTS assay

About 4000 HT1080 cells were seeded in 96 well and incubated overnight for cell attachment. Then cells were treated by HA/Cis/Rb and HA/Rb with HA concentration set at 0, 10.5, 21, 42, 83 and 167 μg/ml. After 3 days of incubation, the cell viability of the cells was evaluated by MTS assay kit (Promega). Briefly, cells were washed and incubated with 100 μl of fresh medium and 20 μl of MTS solution. After 90 minutes of incubation at 37 °C, 100 μl of supernatant was taken out and the absorbance at 490 nm was recorded using plate reader. Absorbance at 630 nm was set as nonspecific absorption and cell viability of control cells was set as 100%.

To probe cytotoxicity of Dasa and Dox-related NGs, around 4000 of MDA-MB-231 and MCF cells in 96-well were incubated with HA/Dasa containing 0.08, 8 and 80 μM Dasa or HA/Dox containing 0.0067, 0.67 and 6.7 μM Dox. Free Dasa or Dox with corresponding concentrations were also included for comparison. After 72h incubation, the cell viability was evaluated using MTS assay.

References

1 E. D. Golla and G. H. Ayres, *Talanta*, 1973, **20**, 199-210.

2 J.-K. Kim, J. Anderson, H.-W. Jun, M. A. Repka and S. Jo, *Molecular pharmaceutics*, 2009, **6**, 978-985.

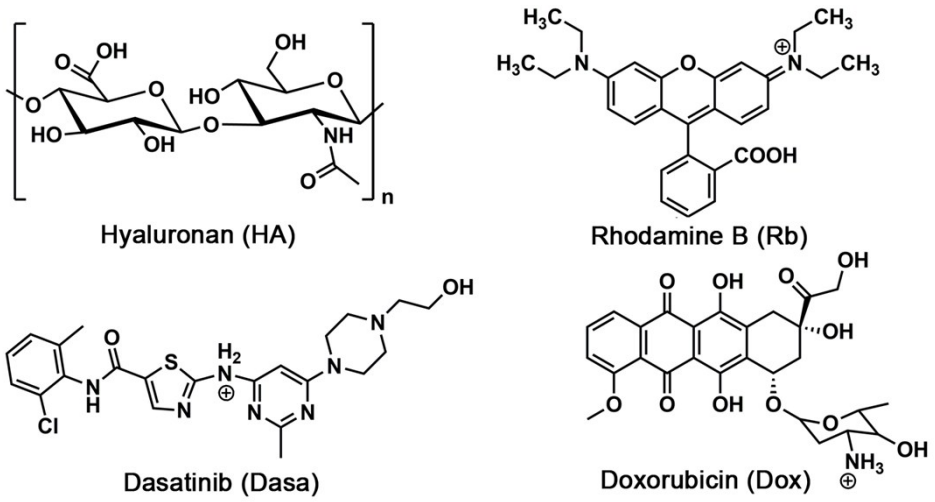

Fig. S1 Molecular structures of HA and drugs tested in cisplatin-mediated NGs.

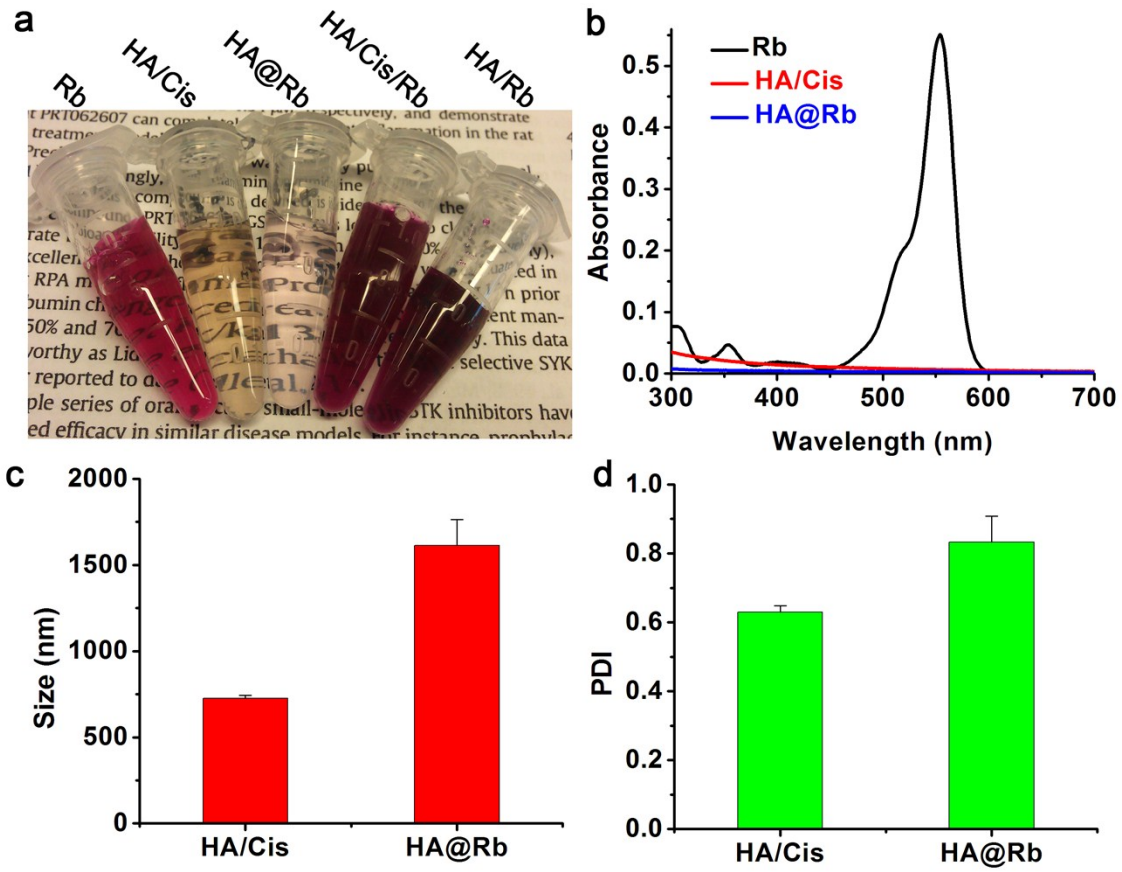

Fig. S2 Characterization of NGs and their related control. a, photograph of Rb solution, HA/Cis, HA@Rb, HA/Cis/Rb and HA/Rb. HA/Cis: HA + Cis dialyzed in H_2O, HA@Rb: HA + Rb dialyzed in H_2O, HA/Cis/Rb: HA + Cis + Rb dialyzed in H_2O, HA/Rb: HA + Cis + Rb dialyzed in PBS. b, Absorption spectra of free Rb, HA/Cis and HA@Rb. HA concentration was set as 0.1 mg/ml. c, Hydrodynamic size of HA/Cis and HA@Rb. d, PDI of HA/Cis and HA@Rb from DLS analysis.

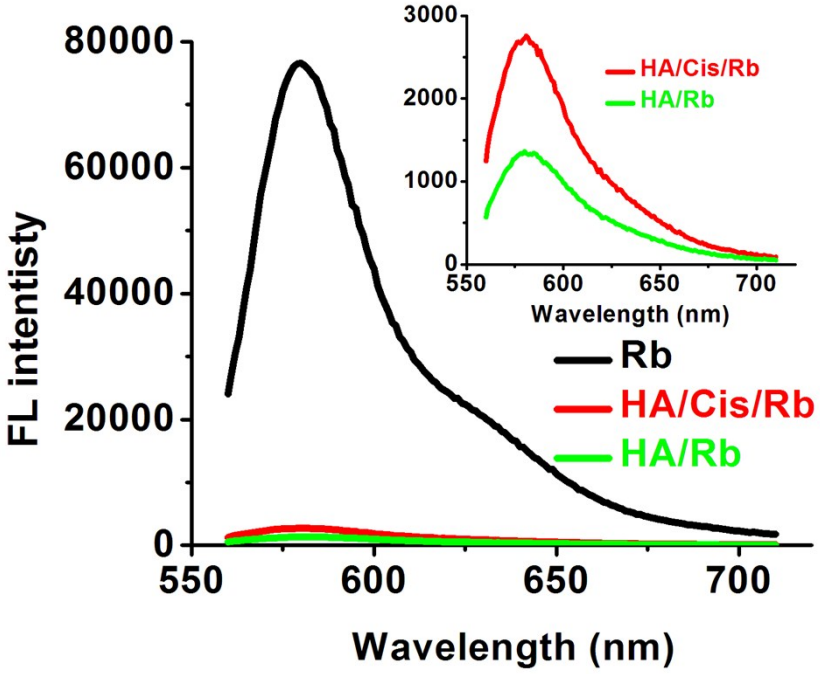

Fig. S3 Fluorescent spectra of free Rb, HA/Cis/Rb and HA/Rb. Rb concentration was fixed at 10 μg/ml.

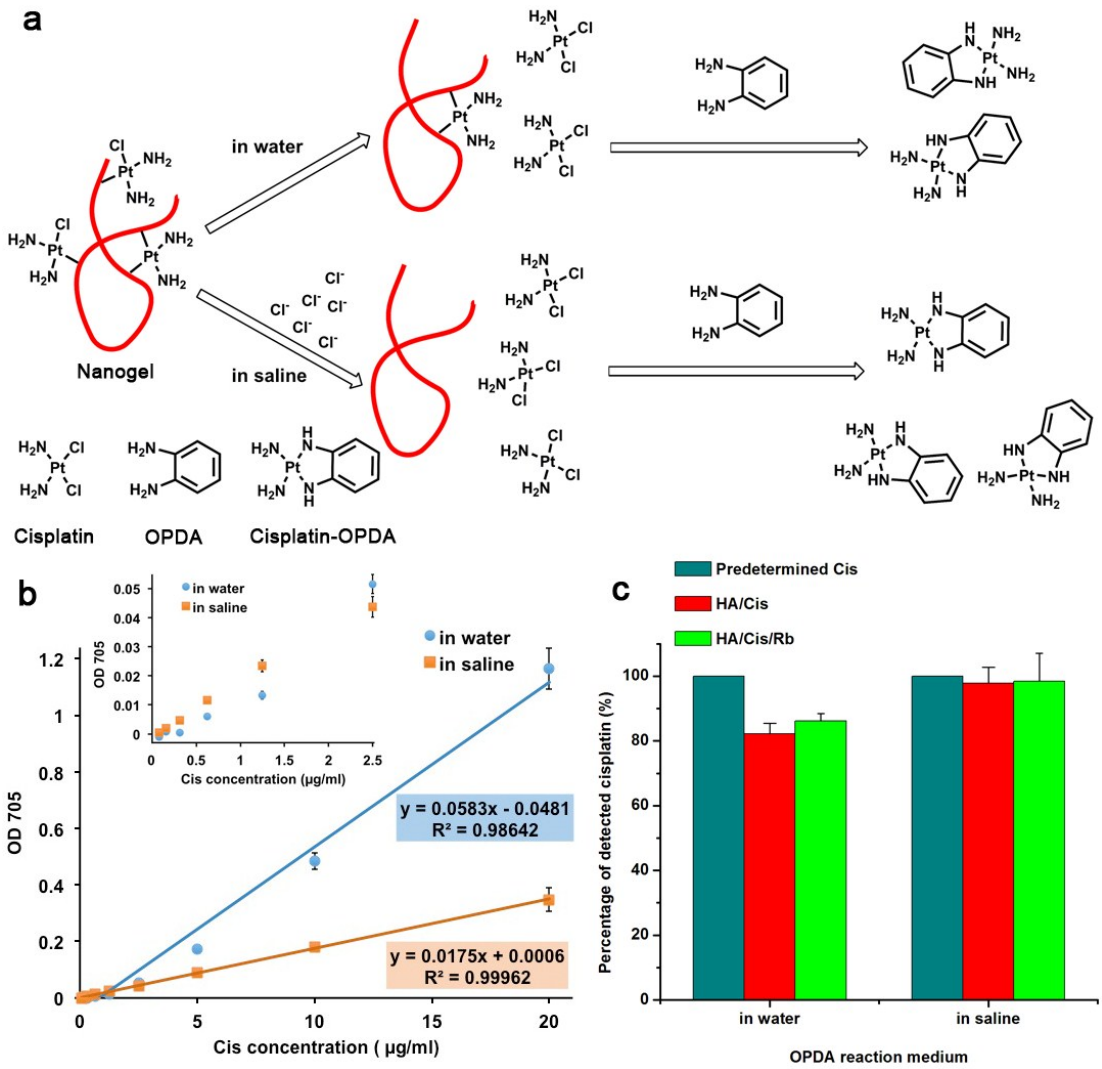

Fig. S4 Modified OPDA method to quantify cisplatin in NGs. a, Schematic illustration of modified OPDA method. Compared with the reaction in water in reported OPDA assay, the reaction was conducted in 9% NaCl. The chloride ion in modified OPDA assay would efficiently detach cisplatin and thus enable the full quantification of cisplatin in NG. b, Standard curve of absorbance of cisplatin-OPDA (OD705) with the cisplatin concentration ranged from 0.08 to 20 μg/ml in water and saline. The presence of chloride suppresses the OPDA reaction, however the linearity is better than that using water as reaction medium. c, Validation of the detection efficacy of modified OPDA assay for NGs. By adding chloride, the modified OPDA method could detect almost all of the cisplatin in NGs.

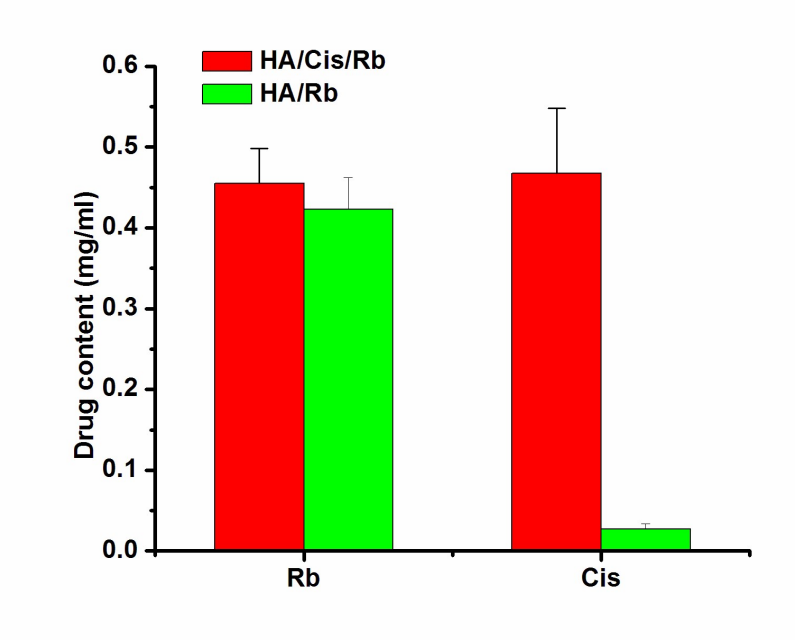

Fig. S5 Drug content in NGs.

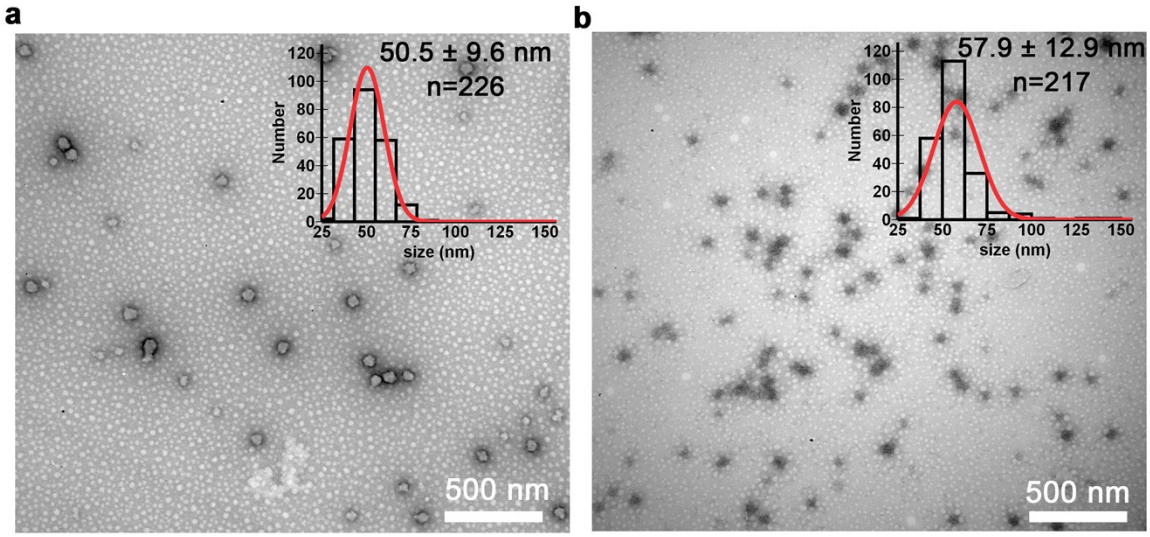

Fig. S6 TEM observation of HA/Cis/Rb (a) and HA/Rb (b). The inserted graph demonstrated the size distribution by counting at least 200 particles from the TEM images.

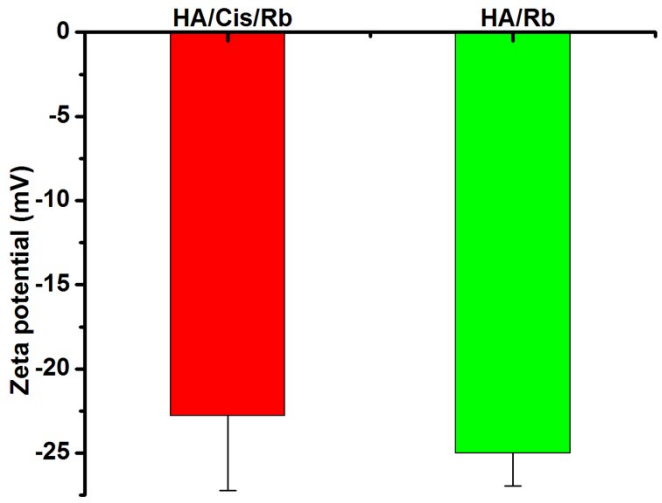

Fig. S7 Zeta potential of NGs.

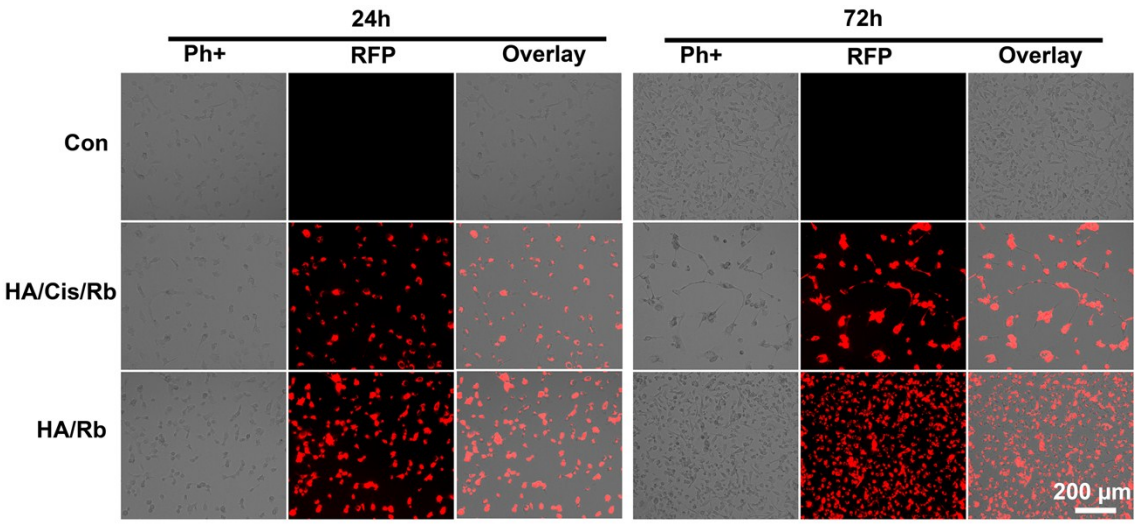

Fig. S8 Fluorescent microscopy of HT-1080 cells after the treatment by NGs for 24 and 72 hours. The concentration of HA/Cis/Rb and HA/Rb were 83 μg/ml in HA. Fluorescent signals of Rb were collected through the RFP channel.

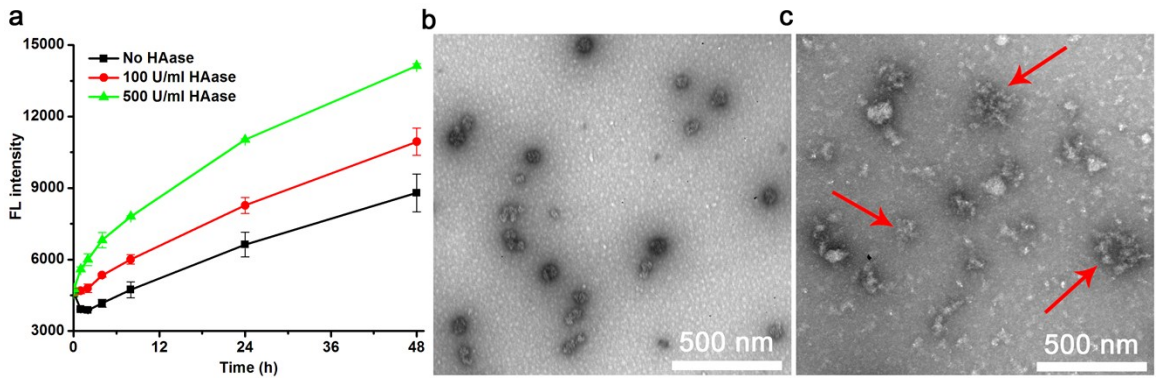

Fig. S9 The degradability of NG in the presence of HAase. a, Rb fluorescence of HA/Rb NG in PBS containing 0, 100, 500 U/ml HAase at 37 °C. b-c, TEM image of HA/Rb after incubation with 0 U/ml (b) and 500 U/ml (c) of HAase for 2 days at 37 °C. The arrows point typical structures of NG debris and disrupted HA/Rb.

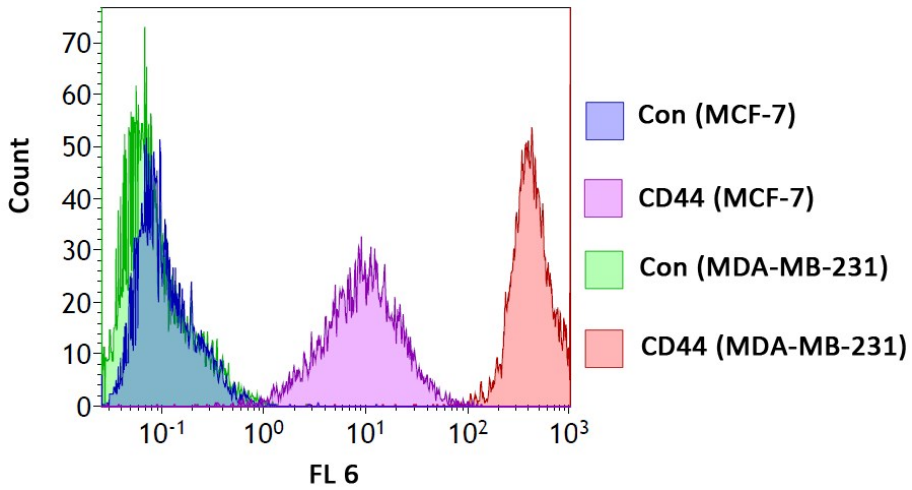

Fig. S10 CD44 expression of MDA-MB-231 and MCF-7 cells from flow cytometer analysis. The cells were labeled by APC-labeled anti-CD44 antibody.

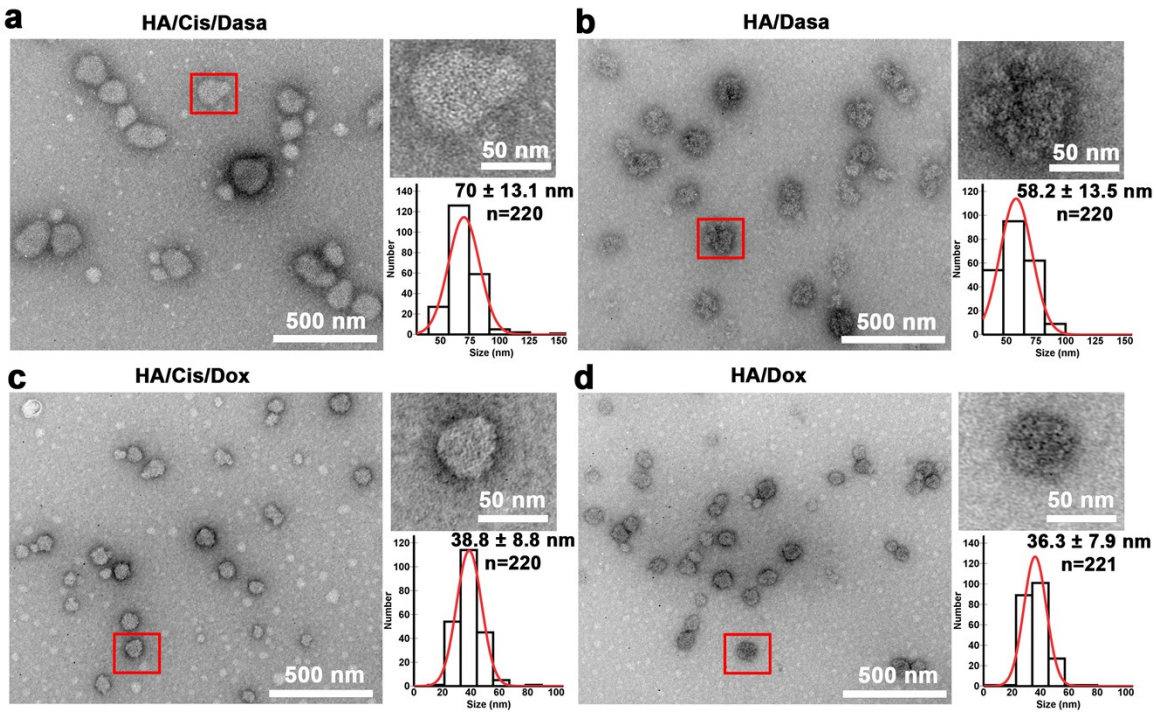

Fig. S11 TEM observation of HA/Cis/Dasa (a), HA/Dasa (b), HA/Cis/Dox (c) and HA/Dox (d).

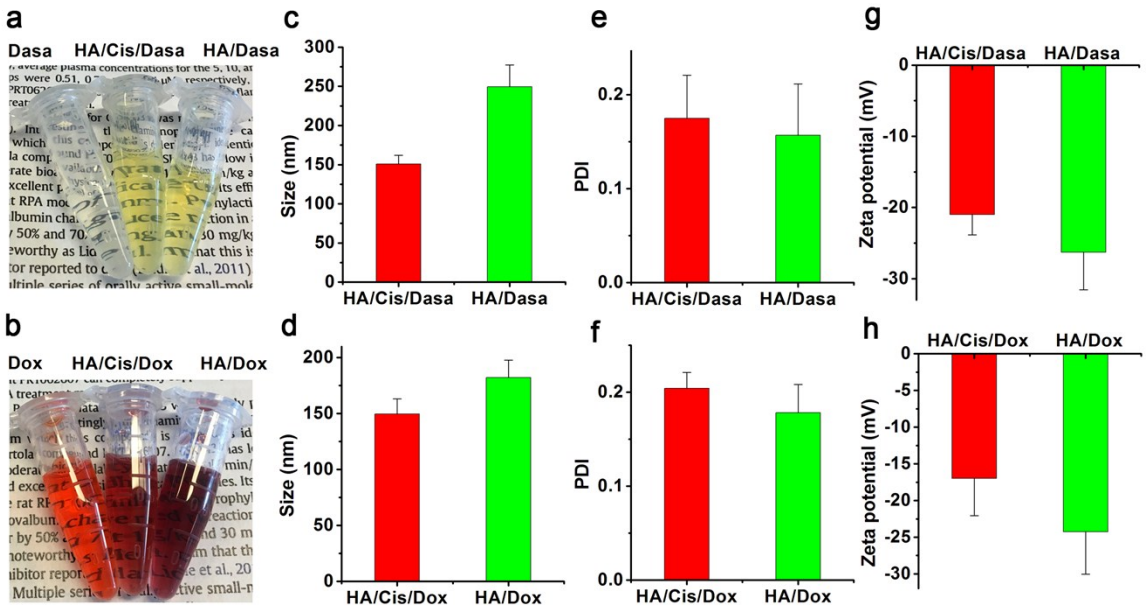

Fig. S12 DLS analysis of Dasa- and Dox-loaded NGs. a-b, photographs of Dasa- and Dox-loaded NGs. Solution of Dasa dissolved in DMF and Dox in water were included for showing the color change of NGs. c-d, Hydrodynamic size of Dasa- and Dox-loaded NGs. e-f, PDI of the Dasa- and Dox-loaded NGs. f, Zeta potential of NGs. After removing the cisplatin, HA/Dasa and HA/Dox demonstrate a swelled size, lower PDI and more negative charges in comparison to the HA/Cis/Dasa and HA/Cis/Dox, respectively.

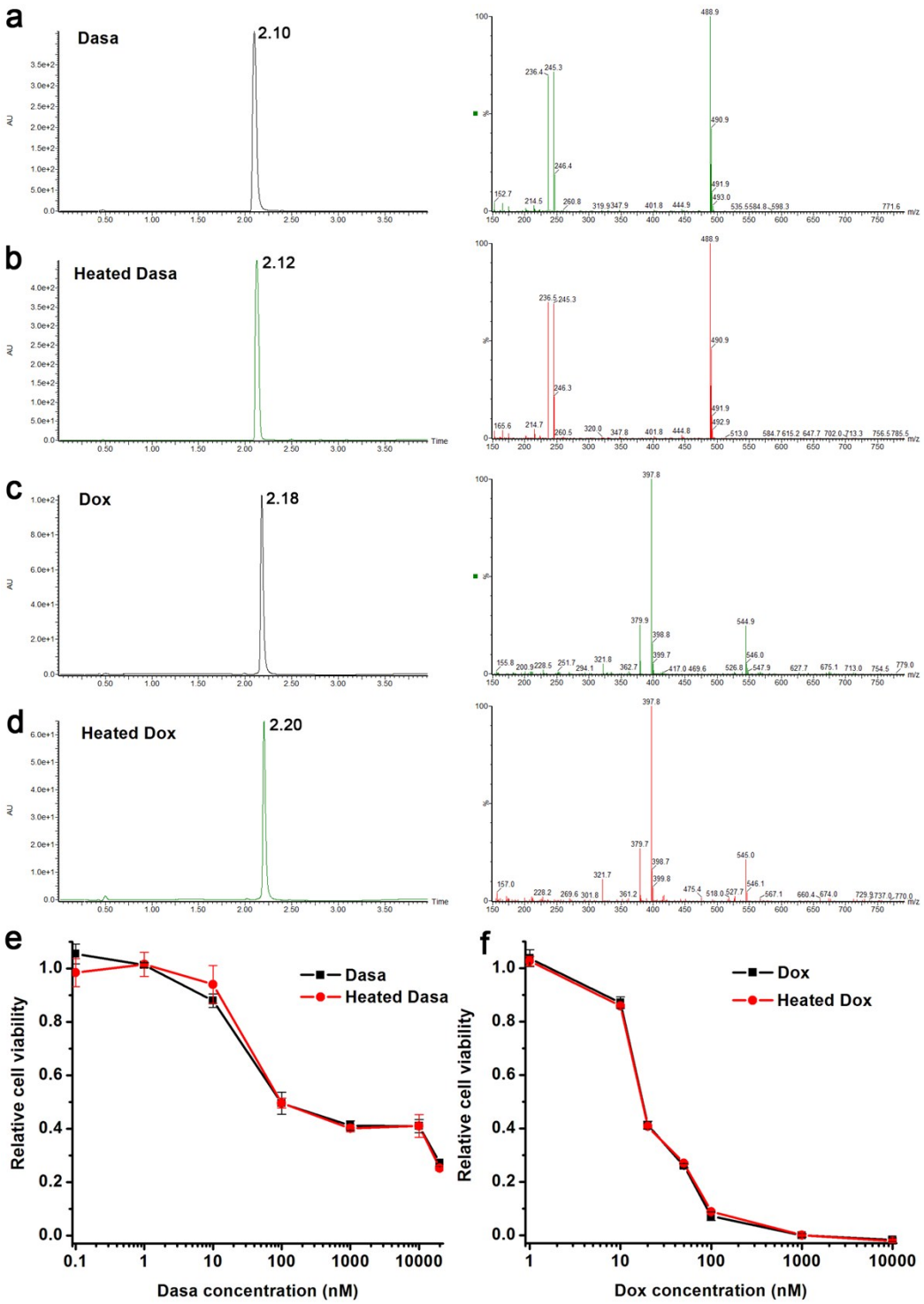

Fig. S13 The integrity and cytotoxic potential of Dasa and Dox after the heating at 90 °C. a-d, The Dasa and Dox were intact after the heating as revealed by LC-MS in comparison to themselves without heat. e-f, The heat didn't affect the cytotoxicity of unheated or heated Dasa and Dox as demonstrated by MTS assay. MDA-MB-231 cells were incubated with different concentrations of unheated or heated drugs for 3 days before the MTS assay.

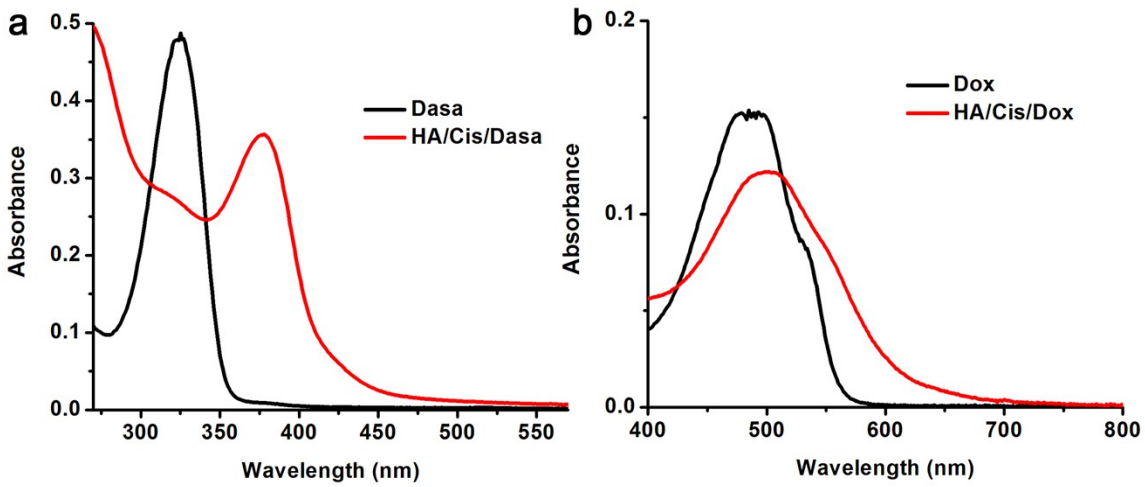

Fig. S14 Absorption spectra of HA/Cis/Dasa (a) and HA/Cis/Dox. Free Dasa and Dox were included to demonstrate the red shift of NGs in absorption.

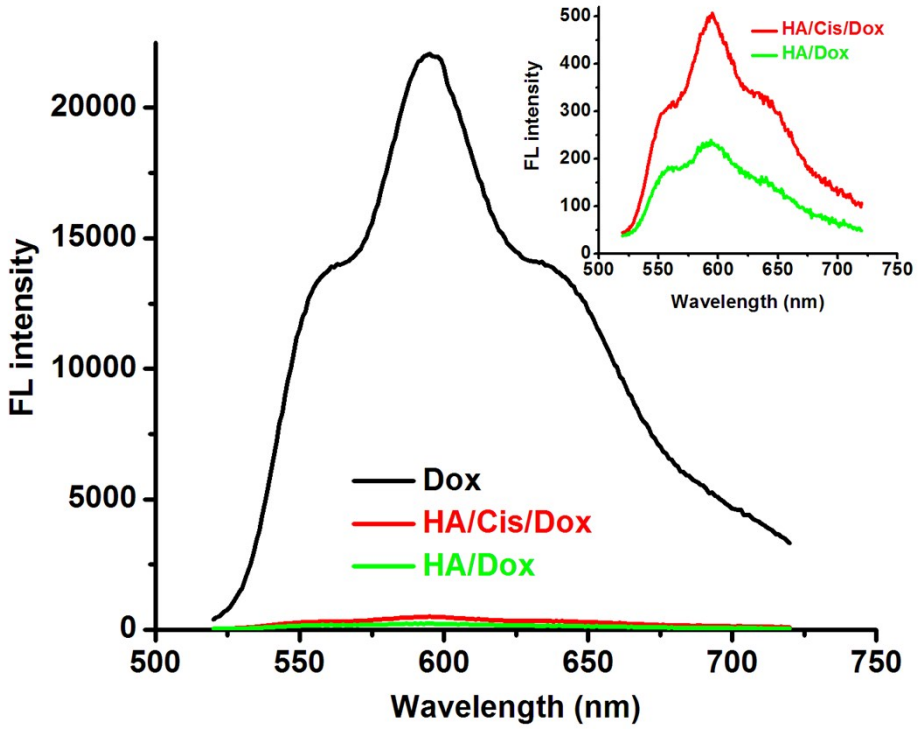

Fig. S15 Fluorescent spectra of HA/Cis/Dox and HA/Dox. Compared with free Dox, Dox in NGs were highly quenched. Dox concentration was set at 40 μg/ml.

Table S1. Drug content in the NGs.

NGs	[Dasa/Dox] (mg/ml)	[Cisplatin] (mg/ml)
HA/Cis/Dasa	0.409 ± 0.024	0.416 ± 0.03
HA/Dasa	0.42 ± 0.028	0.024 ± 0.007
HA/Cis/Dox	0.395 ± 0.007	0.622 ± 0.021
HA/Dox	0.401 ± 0.007	0.037 ± 0.007

Table S2. Loading efficiency of all drugs tested in NGs.

NGs	Rb/Dasa/Dox loading efficiency	Cisplatin loading efficiency
HA/Cis/Rb	10.7 ± 1.02 %	11.0 ± 1.89 %
HA/Rb	11.2 ± 1.04 %	0.71 ± 0.19 %
HA/Cis/Dasa	9.84 ± 0.57 %	10.0 ± 0.71 %
HA/Dasa	11.1 ± 0.75 %	0.63 ± 0.18 %
HA/Cis/Dox	9.08 ± 0.15 %	14.3 ± 0.48 %
HA/Dox	10.6 ± 0.19 %	0.98 ± 0.19 %