Electronic Supporting Information

Overcoming *cis*-platin resistance in chemotherapy by biomineralization

Wei Chen, Yun Xiao, Xueyao Liu, Yanhong Chen, Jiaojiao Zhang, Xurong Xu and Ruikang Tang*

Experimental

Scheme S1. Biomineralization provides a Trojan pathway for *cis*-platin uptake through endocytosis of NSI, avoiding inhibited Ctr1 pathway.

Figure S1. FT-IR Spectrum of NSI

Figure S2. Pt content in NSI *vs* initial *cis*-platin content in solutions

Figure S3. Comparisons of Ctr1 in A549 and A549/DDP cells.

Figure S4 *In vitro* toxicity of *cis*-paltin and biomineralized *cis*-paltin for MG63 and HepG2.

Figure S5. Carbonated calcium phosphate particles resulted by DMEM biomineralization and their toxicity.

Figure S6. Remained FITC-BSA in solution after *cis*-platin biomineralization **Figure S6.** NSI internal-ization.

Figure S7. NSI internalization.

Figure S8. Solution stability of NSI.

Figure S9. Animal tests using *cis*-platin-sensitive A549 tumour bearing nude mice.

Table S1. Composition of the low Cl^- DMEM

Experimental

Materials. All chemicals including *cis*-$Pt(NH_3)_2Cl_2$ were purchased from Sigma-Alderich (USA). The cell culture media RIPM 1640, trypsin and FBS, amino acids as well as vitamins were obtained from Gibco (USA). DAPI, Lyso-tracker Red, FITC and BCA assay were provided by Beyotime (China).

Low Cl^- DMEM. The solution composition was the same to the normal DMEM except that Cl^- was replaced by NO^{3-} and the detailed information was listed by Table S1. The solution pH was adjusted to 7.2 by 1 M NaOH.

Cells. A549 cell lines were purchased from American Type Culture Collection (USA) and maintained in RPMI 1640 media supplemented with 10% FBS at 37°C in a 95% humidified atmosphere with 5% CO_2. The *cis*-platin resistant A549/DDP cell lines were purchased from Aiyan Biological Technology (China) and then frozen and stored in liquid nitrogen. Fresh aliquots of A549/DDP were used in all experiments and the cells maintained in the cell culture with 2 μg/ml *cis*-platin before treatment to ensure that A549/DDP did not revert to the drug sensitive phenotype. The low expression of Ctr1 of A549/DDP was confirmed by RT-PCR and western blot analysis.

RT-PCR. Total RNA in samples was extracted using RNeasy Mini kit (Qiagen, USA) following the manufacturer's protocol. 600 ng obtained RNA was reverse transcripted into complementary DNA (cDNA) using CX100 Droplet Digital^{TM} PCR (Bio-Rad, USA). 1 μl cDNA was subjected to the RT-PCR analysis using the SYBR Premix Ex Taq (Takara, China). Quantitative analysis was performed by an IQ^{TM}5 real-time PCR detection system (BioTek, USA). Relative gene expression values were determined by the $2^{\Delta\Delta CT}$ method with human S18 gene as the endogenous reference. The following sequences were used as the PCR primers:

Ctr1-forward, 5'-GCCTATGACCTTCT ACTTTGGCTT-3';

Ctr1-reverse, 5'-CTCGGGCTATCTTGAGTCCTTC-3';

18S-forward, 5'-GA CTCAACACGGGAAACCTCAC-3';

18S-reverse, 5'-CCAGACAAATCGCTCCACCAA C-3'.

Western Blot. 5×10^4 A549 or A549/DDP cells were washed using PBS and they were re-suspended in 50 μl lysis buffer (containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, and1 mM EDTA; solution pH of 7.5) supplemented with Roche's Complete Protease Inhibitor Cocktail Tablets. 50 μg cellular proteins was separated by 12% SDS-PAGE electrophoresis and then transferred onto nitrocellulose filter membranes (Millipore, USA). Non-specific binding sites of the membrane were blocked using 5% BSA in PBS, and probed with the monoclonal antibody against Ctr1 (1:1000, Santa Cruz, USA) following by HRP conjugated goat anti-mouse antibody (1:10000, Santa Cruz, USA). Signals were visualized using the enhanced chemiluminescence (ECL) system (Thermo, USA). The expression level of Ctr1 protein was normalized to the β actin protein expression level.

Preparation of biomineralized *cis*-platin. The modification is simple just like the Akaike's method.[1] Typically, *cis*-platin (0-33 μM) was equilibrated in 2 ml Dulbecco's Modified Eagle's Medium (DMEM) for 24 h. Since chloride ions inhibit the hydrolysis and substitution of PO_4^{3-} or CO_3^{2-} to Cl^- of *cis*-platin, the low chloridion DMEM (Cl^- was replaced by NO_3^-, Table. S1) was used to facilitate the intermedia formation. The *in situ* biomineralization was initiated by an addition of 20 μl 1.0 M $Ca(NO_3)_2$ into the *cis*-platin solution at 37°C in a 95% humidified atmosphere with 5% CO_2. After 4 h, the solution modification was terminated by 200 μl fetal bovine serum (FBS) due to its inhibitory effect on biomineralization.[2]

Characterizations. TEM and EDS examinations of NSI were performed by a JEM-1200EX (JEOL, Japan) and a JSM-35CF (JEOL, Japan), respectively. FT-IR was examined by a Nexus 670 (Nicolet, USA). A Nano S Zetasizer (Malvern, UK) was used to measure the DLS size distribution of NSI in the

biomineralized solution.

ICP-MS. Pt content in *cis*-platin solutions was examined by a XSENIES (Thermo, USA). After biomineralization, the isolated NSI particles (centrifugation at 10,000 rpm, 10 min) were dissolved in 1×10^{-6} M HCl solution and Pt content was also examined. Thus, the biomineralized percentage of *cis*-platin could also be estimated. Besides, calcium contents in the NSI solid phase were also examined.

Uptake. A549 and A549/DDP cells at a density of 5×10^4 cells/well were seeded in the 3.5 cm culture flast and maintained at 37°C in a 95% humidified atmosphere with 5% CO_2 for 24 h. After 2 h incubation with the *cis*-platin or its modified solutions (the Pt concentration was fixed at 10 μM), the cells were washed with 500 μl PBS, digested, counted and collected. After exposing cells in the Radio Immunipricipitation (RIPA) Assay lysis buffer (Beyotime, China), the supernatant (cells) was separated by a centrifugation at 1500 rpm for 5 min. The total Pt and total protein contents in the supernatant were determined by ICP-MS and BCA, respectively. The total protein content could be used as a standard expression for the cell numbers and the intercellular Pt concentration was expressed as nanograms of Pt per microgram of protein. Each experiment was repeated three times and the average values were taken in the analyses.

NSI stability. NSI particles were re-dispersed to water. The initial solution pH was 7.6 and then the value was decreased using 1M HCl. The resulted NSI phase had a characteristic absorption band at 320 nm and the absorbance, OD_{320}, had a positive correlation with the particle amount. OD_{320} of the solution at different pH was measured by an Eon Microplate Spectrophotometer (BioTek, USA).

FITC-BSA. 500 mg BSA was added to 2 ml of 0.25 M bicarbonate buffer (pH = 9.8). 9 mg FITC was dissolved in 100 μl DMSO and then, the solution was added to the BSA solution. The reaction solution was stirred slowly at 4°C overnight. Fluorescent-labelled BSA was dialyzed exhaustively (8,000 Da

molecular weight cutoff) against bicarbonate buffer (0.25 M, pH = 9.8).

Fluorescent NSI. 10 μM *cis*-platin was balanced in 2 ml low Cl^- DMEM and then 50 μl FITC-BSA (1 mg/ml) was added. The biomineralization modification was triggered by an addition of calcium and the FITC-BSA-NSI particles were obtained in the solution.

Cytophagy. FITC labelled BSA was introduced into the *cis*-platin solution (1 mg/ml 50 μl) prior to the biomineralization modification. After the modification, the biomineralized solution was incubated with cells for 3 h. The cells were stained by Lyso-Tracker Red according to the protocol, washed with PBS, fixed using cell fixing solution (4% paraformaldehyde, 4% NaOH, 4% sucrose, 1.68% NaH2PO4, pH=7.5-8.0) for 30 min at room temperature. Nuclei were stained by DAPI according to the protocol. Each sample was observed direct by using a LSM-510 confocal laser scanning microscope (Zeiss, Germany).

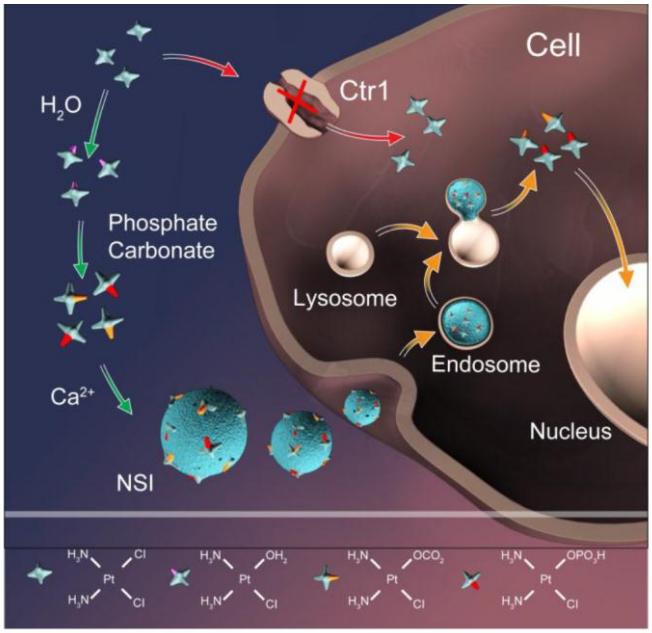
Cytotoxicity. A549 and A549/DDP cells (1×10^4 cells/well) were seeded in 96-well plate respectively in 200 μl culture media and incubated for 24 h before the drug treatment. Meanwhile, *cis*-platin was balanced in low Cl^- DMEM (500 μl) in a 24-well plate for 24 h. 100 μl *cis*-platin (0.00, 2.08, 3.13, 4.17, 6.25, 8.33, 12.50, 16.67, 25.00, 33.33μM) solution or the same dose of biomineralization modified solution was added to the cells and the incubate period was 48 h. After 4 h incubation with the addition of 20 μl 5 mg/ml MTT each well, the media were removed and cells were washed with PBS for three times. 150 μl DMSO was added and the absorption (570 nm) was measured by Eon Microplate Spectrophotometer (BioTek, USA), which was positive correlation to concentration of formazan that represented the activity of succinodehydrogenase. Cell viabilities at various Pt concentrations were presented as the percentage of the control group without any drug treatment. Each experiment was repeated three times and the average values were taken in the analyses. The IC50 values were calculated using SPSS 8.0 software. MG63 and HepG2 cell lines were tested in the same way.

Animal test. The experiments were performed by the Laboratory Animal Centre of Zhejiang University. All animals were treated ethically and followed the protocols approved by Zhejiang University. All mice were purchased from Slac (China). The A549 and A549/DDP tumour models were generated by subcutaneous injection of 5×10^6/ml cells in 100 μl DMEM into the left shoulder of 3-week-old female nude mice (18 g). The mice were used for treatment when the tumour volume reached 50 to 100 mm^3 (around 2 weeks) on average and the day was recorded as day 0. For the treatment the mice were divided into 3 random groups. The tumour therapy consisted of administration of 100 μl *cis*-platin or the biomineralized solution (dose of *cis*-platin was 1.25 mg/kg). 100 μl low Cl^- DMEM was used as a control. The treatments performed by injecting the agents into the tumour-bearing mice at tail vein once every 4 days. The mice were observed daily for clinical symptoms. The tumours were measured for the longest diameter (L) and the shortest diameter (B) using a calliper and their volumes were calculated by the formular, $0.5\times L\times B^2$. At day 16, the tumours were harvested and weighted immediately from the sacrificed mice. The tissues were fixed in 10% formalin for conservation. To calculate the value of TGI, the tumour volumes on day 16 were recorded and calculated by the equation below:

Tumour growth inhibition (TGI)= $1-(V_{16}/V_0)_{experimental}/(V_{16}/V_0)_{control}$

where V_0 = the volume of the tumour on day 0, V_{16} =the volume of the tumour on day 16.

1. S. Tada, E. H. Chowdhury, C. Cho and T. Akaike, *Biomaterials*, **2009**, 31, 1453-1459.
2. P. Liu, J. Tao, Y. Cai, H. Pan, X. Xu and R. Tang, *J. of Cryst. Growth.*, 2008, **310**, 4672-4675.



Scheme S1 Biomineralization provides a Trojan pathway for *cis*-platin uptake through endocytosis of NSI, avoiding inhibited Ctr1 pathway.

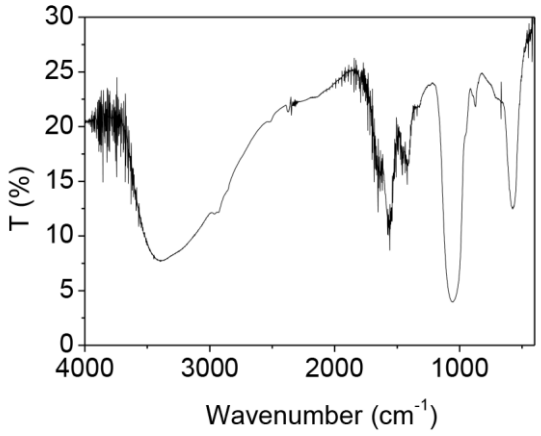


Figure S1 FT-IR Spectrum of NSI. The broad peak around 3400 cm^{-1} may include the signal of amino group; the peaks between 1410 and 1540 cm^{-1} are assigned to carbonate group, and the bands at 880 cm^{-1} and 1000-1100 cm^{-1} are due to P-O bending and stretching modes, respectively.

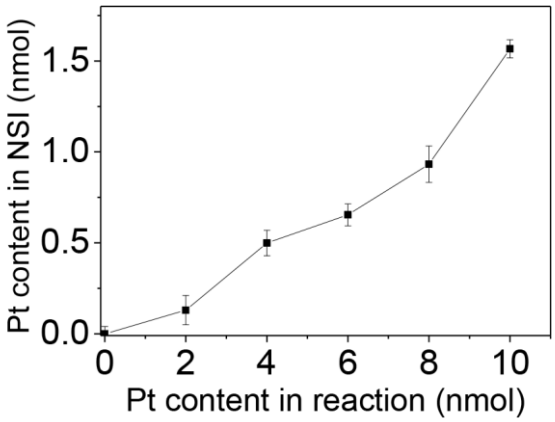


Figure S2 Pt content in NSI *vs* initial *cis*-platin content in solutions. The curve indicates that about 10% of the Pt compounds turned into the NSI phase by the *in situ* biomineralization.

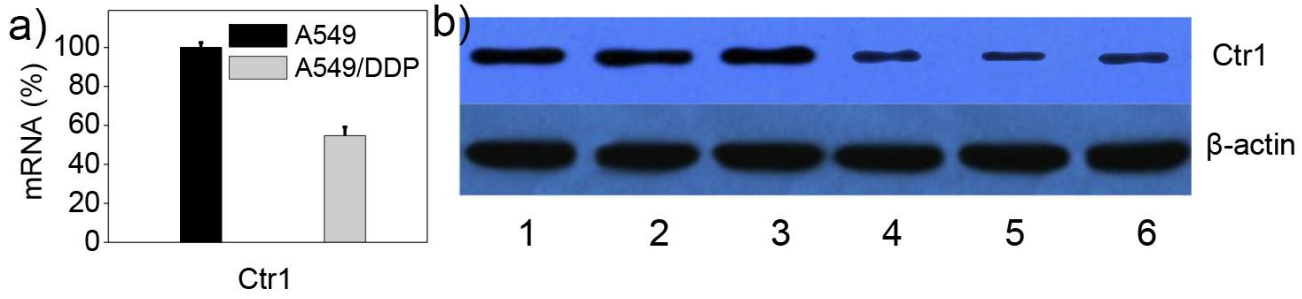


Figure S3 Comparisons of Ctr1 in A549 and A549/DDP cells. (*a*) mRNA levels of Ctr1 measured by real-time PCR. (*b*) Western blot analysis. Each test was repeated for 3 times. Lane 1, 2, 3 for A549, lane 4, 5, 6 for A549/DDP. These results confirmed that the drug resistance of A549/DDP was caused by the Ctr1 deletion.

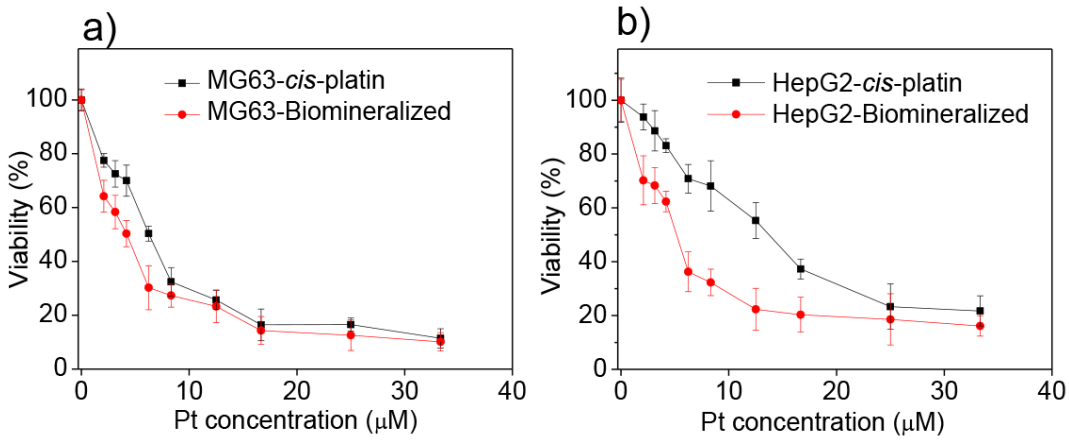


Figure S4 *In vitro* toxicity of *cis*-paltin and biomineralized *cis*-paltin for (*a*)MG63 and (*b*)HepG2.

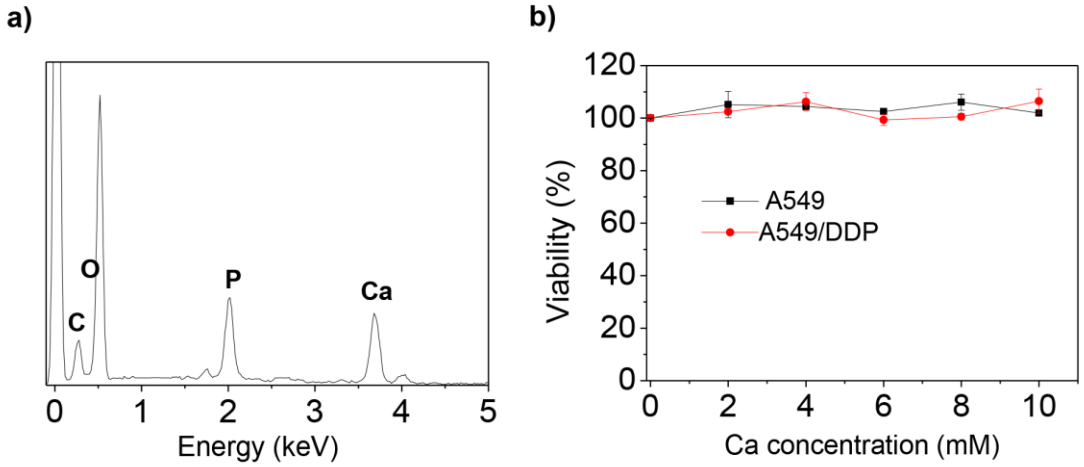


Figure S5 Carbonated calcium phosphate particles resulted by DMEM biomineralization and their toxicity. (*a*) EDS result showed that the resulted solid was a kind of calcium phosphate with carbonate and there was no Pt. (*b*) Accordingly, these particles did not exhibit any cytotoxicity in both A549 and A549/DDP. (Note: the precipitated particle amounts were related to the extra added calcium concentration)

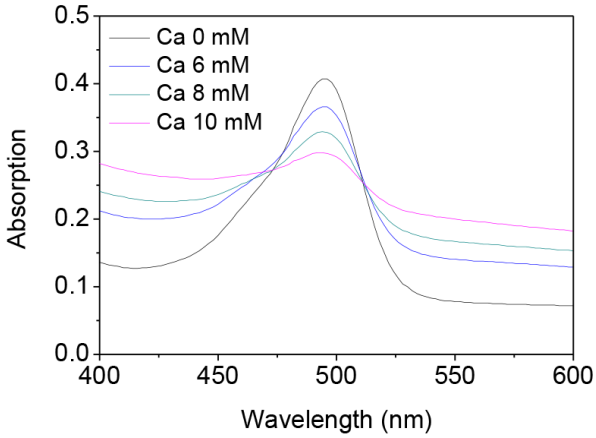


Figure S6 Remained FITC-BSA in solution after *cis*-platin biomineralization. The absorption of supernatant decreased with the increasing of calcium concentration, implying the integration of FITC-BSA into NSI. The mineralization in DMEM was triggered by the extra calcium ions.

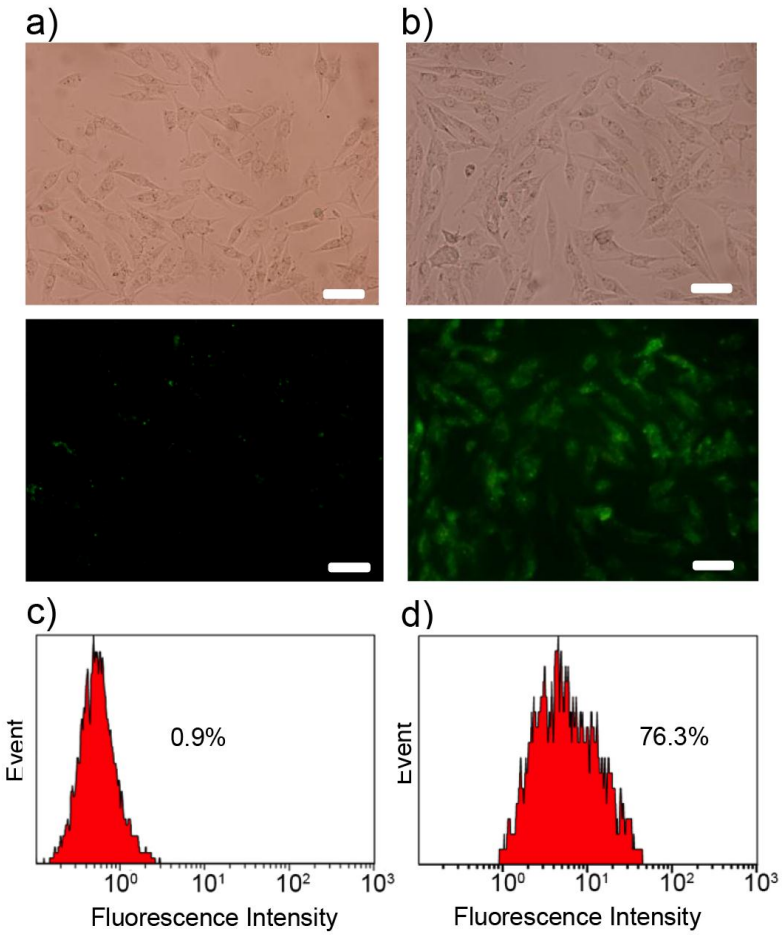


Figure S7. NSI internalization. (a) Micrographs of A549/DDP cells treated by FITC-BSA. No internalization was observed (bar = 10 μm). (b) Micrographs of A549/DDP cells treated by biomineralized cisplatin in the presence of FITC-BSA. The internalization was observed at large scale. Up, the cells under bright field; down, the FITC-BSA (green) in the cells under 450-480 nm excitation. (c) Flow analyses of the FITC-BSA uptake by A549/DDP. (d) Flow analyses of the FITC-BSA-NSI uptake by A549/DDP.

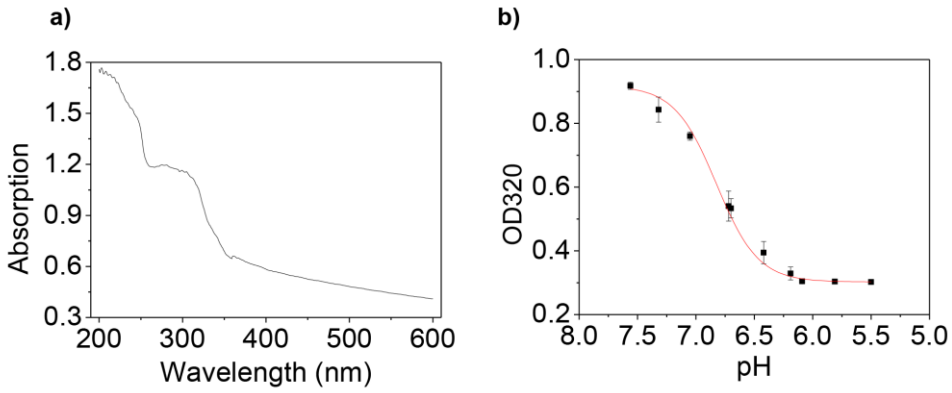


Figure S8 Solution stability of NSI. (*a*) UV-VIS spectrum of the biomineralized *cis*-platin solution. The specific adsorption around 320 nm was attributed to the NSI formation. (*b*) Curve of OD_{320} *vs* solution pH. Clearly, the stability of NSI decreased with the pH decreasing.

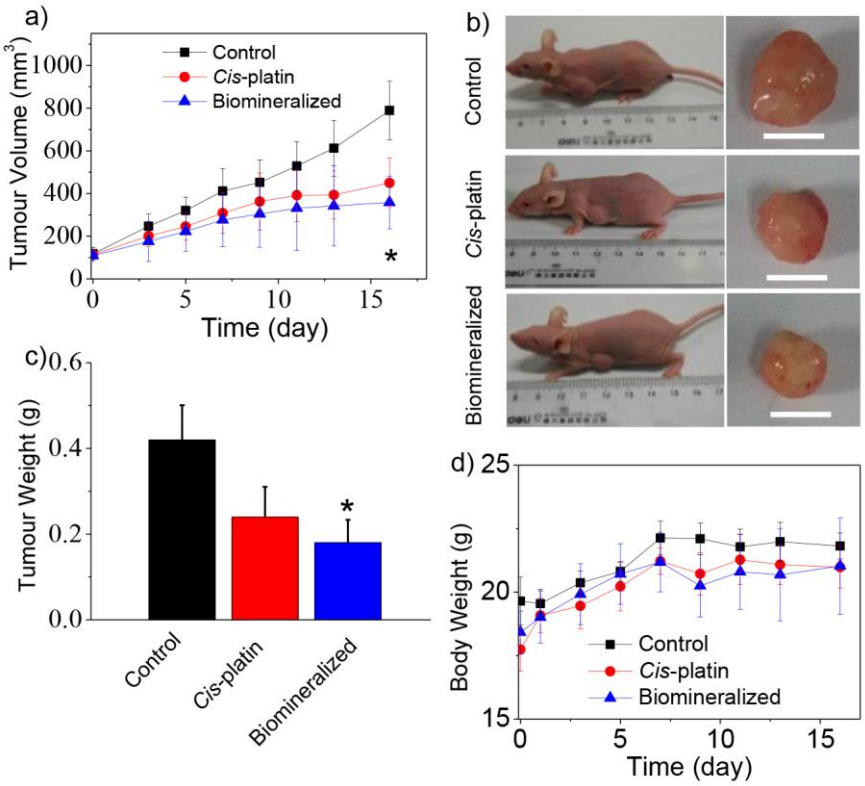


Figure S9 Animal tests using *cis*-platin-sensitive A549 tumour bearing nude mice. (*a*) Growth curves of A549 tumours treated by *cis*-platin and biomineralized *cis*-platin. (*, $p<0.05$). (*b*) Representative photos of mice from different groups were taken at the end of treatment on day 16 (Scale bar = 1 cm). (*c*) Statistics of tumour weights on day 16 (*, $p<0.05$). (*d*) Weight curves of mice during the treatments. There was no significant difference among the three groups.

Table S1. Composition of the low Cl^- DMEM.

Compound	**Content (mg/l)**	**Compound**	**Content (mg/l)**
$Ca(NO_3)_2$	425.57	L-Methionine	30.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.10	L-Phenylalanine	66.00
KNO_3	542.45	L-Serine	42.00
$Mg(SO_4)_2$	97.67	L-Threonine	95.00
$NaNO_3$	9307.60	L-Tryptophan	16.00
NaH_2PO_4	125.00	L-Tyrosine	104.00
Glucose	4500.00	L-Valine	94.00
$NaHCO_3$	3696.44	D-Calcium Panto-thenate	4.00
L-Arginine	84.00	2-Hydroxyethyl trimeylammonium chloride	4.00
L-Csteine	63.00	Folic acid	4.00
L-Glutamine	584.00	Inositol	7.20
Glycine	30.00	Nicotinamide	4.00
L-Histidine	42.00	Riboflavin	0.40
L-Isoleucine	105.00	Thiamine	4.00
L-Leucine	105.00	Vitamin B6	4.00
L-Lysine	146.00	Sodium Pyruvate	110.00