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Cancer Nanomedicine: From PDGF Targeted Drug Delivery

Chandrababu Rejeeth[#]*, Raju Vivek[#] , Varukattu Nipun Babu and Alok Sharma[#]

Proteomics & Molecular Cell Physiology Laboratory, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore 641 046, Tamilnadu, India

[#]*Current address: Department of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China.*

Experimental

Materials

cis-Diamminedichloroplatinum(II) (cisplatin), succinic anhydride, fluorescamine, tetraethylorthosilicate (TEOS), (3-aminopropyl)- triethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide - HCl (EDC), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)□fluorescein isothiocyanate (FITC) Sodium sulfite (Na_2SO_3) and Pluronic P-123 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethyl alcohol (absolute), dimethyl sulfoxide (DMSO), N, N-dimethyl formamide and hydrogen peroxide (34.5%) were purchased from Samchun Chemicals (Korea). N-Hydroxysuccinimide (NHS), chlorin e6 (Ce6) was purchased from Fluka (China).

PDGF binding studies

For the protein pull-down studies, 1 μg of fluorescently labeled PDGF in 100 μL of PBS was incubated with increasing amount of the nanoparticles. The incubation was carried out at 37 °C for 5 min followed by centrifugation at 50 000g for 30 min. The amount of PDGF in the supernatant was then determined by fluorescent spectroscopy (POLAR star, BMG Lab tech). Protein-only controls showed that no PDGF pelleted under the centrifugation conditions. All experiments were performed in triplicate.

A trace level of background fluorescence in the supernatants was seen during the pull-down experiments, which was due to residual dye after desalting the samples. This background signal was subtracted from all of the fluorescence measurements when calculating the PDGF amounts. Nonspecific adsorption of PDGF to the tubes during the incubation was determined using a PDGF-only control. On the basis of the control

experiments, the free PDGF was approximately 0.7 μg (i.e., 7 μg/mL), which was taken into account to calculate the total amount of available PDGF. After centrifugation, the amount of nanoparticle-bound PDGF was calculated by subtracting the amount of PDGF measured in the supernatant from the total PDGF.

The amount of bound PDGF (pmol) was plotted against the concentration of the PAA-MSNs (nM). The molarity of the PAA-MSNs and PDGF was determined using the nanoparticle density of 19.3 g/cm3 and PDGF molecular weight of 14.4 kDa. A binding saturation curve was fitted to the protein pull-down data. The binding affinities and maximal binding capacity were determined by nonlinear least-squares regression analysis (Prism 5, GraphPad Software) using the equation

$$\mathbf{B} = \frac{Bmax[NP]h}{Kdh + [NP]h}$$

where B is bound PDGF, Bmax is the maximum binding capacity, [NP] is the concentration of PAA-MSN, Kd is equilibrium binding constant, and h is the Hill coefficient.

For the competition assay, a slight excess amount of each nanoparticle (60 nm = 17.7 nM; 70 nm = 16 nM; 80 nm = 8.2 nM; 90 nm = 5.2 nM; 100 nm = 4 nM) was incubated with 1 μg of fluorescently labeled PDGF at 37 °C for 5 min. After the initial incubation, 100-fold excess unlabeled PDGF was added and incubated at 37 °C for up to 240 min. At different time points, individual samples were centrifuged, and the amount of the labeled PDGF in the supernatants was determined as described above. Separate samples were used at each time point. A one-phase exponential decay curve was used to model the dissociation of the PDGF from the nanoparticle surface. The dissociation rate constants, Koff, of the protein-nanoparticle complexes were determined by fitting the equation to the data:

$$Y = 100e^{-K_{off}t}$$

where Y is the bound PDGF (% total), t is time (min), and Koff is the dissociation rate constant (min^{-1}). All of the experiments were performed in triplicate.

Synthesis of Pt (IV)-based prodrug

The c,c,t-[$Pt(NH_3)$ $2Cl_2$ $(O_2CCH_2CH_2CH_2CO_2H)_2$] (Pt(IV) prodrug) was prepared according to the previous reports.[1] Briefly, cisplatin was oxidized by hydrogen peroxide in

water at 50º C for 1 h and recrystallized with cold acetone. This oxidized cisplatin, c,c,t-[$Pt(NH_3)2Cl_2(OH)_2$], was reacted with succinic anhydride in DMF at 75º C for 4 h. The product was dried under vacuum, and this dark yellow oil was dissolved in acetone and precipitated in cold ether. The product was obtained as a yellow solid at 50% yield.

Synthesis of mesoporous silica nanoparticles MSN

The nanomaterials intricate in this work were mesoporous silica nanoparticles (MSN) and modified MSN. The MSN synthesis was reported some minor modification.[2] First, 1 g of Poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (EO20PO70EO20) copolymer (typical Mn 5800, denoted P123) as a template was stirred with anhydrous sodium sulfate (1.2 g) in a buffer solution (HAc-NaAc, pH=5, 0.2 M, 25 g) at room temperature (25° C) for 12 hr. The solution was then heated to 40° C. Tetraethyl orthosilicate (TEOS, 1.5 mL) as a silica source was added into the P123 solution under stirring and the mixed solution was kept in static condition for 24 hr. After that, the crude product was transferred to an agitated reactor for another 24 hrs hydrothermal reaction at 100° C. In the fourth step, the purified MSN products were baked at 500 °C for 5 hr.

For the amino-modification of MSN (MSN- NH_2), 1.5 mg of calcined MSN was dried and degassed at 110° C, and then suspended in 30 g of waterless toluene. To control the reaction rate, 2 mL of (3-aminopropyl) trimethoxysilane (APETS) was slowly added into the above slurry under stirring when the temperature was raised to 110° C. The slurry was stirred and refluxed at 24 hr. The resulting solid was collected and washed by toluene, dichloromethane, and ethanol, sequentially, and then dried at 70 °C.

Synthesis of Cisplatin prodrug-MSN conjugates

For a typical amide coupling reaction, prodrug, c,c,t-[$Pt(NH_3)2Cl_2(O_2CCH_2CH_2CH_2CO_2H)_2$] (0.8 mM) was reacted with 1 mM NHS and 1 mM EDC for 10 min at room temperature in DMSO (6 ml), and then it was added to well-dispersed amine functionalized MSN@NH_2 (0.2 mM) in DMSO (6 ml). The mixture was stirred at room temperature for 1 day, followed by centrifugation and sequential washing with DMSO, a water/methanol mixture and water. Cisplatin prodrugs to measure the amount of Pt in the prodrug, a previously reported Pt quantification method was adopted, modified and applied in this study.

Synthesis of Poly acrylic acid coted cisplatin prodrug conjugates MSN

MSN@NH_2@cis-DDP (30 mg) was dispersed in 10 mL of DMF, and then 10 mg of PAA (Mw = 1800) was dissolved into the mixture. The reaction mixture was stirred at 140° C for 2 h. After the reaction, the mixture was centrifuged and washed with copious ethanol. To ensure that the PAA physically adsorbed on MSN was removed completely, the washing procedure was repeated until the weight loss of PAA-MSN did not change. The resultant product was dried overnight in a vacuum at 45º C.

General Instruments

The fluorescence emission spectra were obtained using a Shimadzu RF-5301PC spectrofluorophotometer. 1H NMR spectra were recorded on a Bruker AVANCE-400 NMR spectrometer. The transmission electron microscope (TEM) images and energy-dispersive spectrometry (EDS) were taken on a JEOL JEM-200CX transmission electron microscope. Pt content was measured on a Thermo X series 2 inductively coupled plasma-mass spectrometer (ICP-MS). Confocal laser scanning fluorescence microscopy images were obtained with a Zeiss LSM-710 microscope.

Cell culture

Human cervical cancer (HeLa) cell line was obtained from National Centre for Cell Science (NCCS, Pune, India). The cells were maintained at 37 °C fewer than 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) high glucose, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 lg/ml) and L-glutamine (4 mM). For cell viability testing, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA).

In vitro drug release experiment

MSN@cis-DDP nanoparticles and MSN@cis-DDP/PAA nanocomposite were suspended, respectively, in 4 ml PBS buffer (pH=7. 4) with a concentration of 0.1 mg/ml at 37° C under constant stirring. The solution pH was adjusted by titration of 1.0 M HCl to achieve pH values of 5. After incubation for 1, 2, 3, 5, 8, 12, 24, 36, 48 and 72 hours, the nanoparticle suspensions were centrifuged at 3000 rpm to isolate the nanoparticles from the released cis-DDP and PBS solutions. The released cis-DDP was analyzed by monitoring the absorbance at 482 nm, and the amount was determined by the calibration

curve of cis-DDP in PBS (C μg/ml = 42.7758Abs + 0.2244), range from 0 to 25 μg/ml with R^2 =0.9999.

Intracellular uptake of the MSN@cis-DDP@PAA nanocomposite

The HeLa cells were cultured in 12-well plates (1×10^5 cells per well) overnight, then the culture medium were replaced with fresh culture medium containing drug carrier particles and incubated in a fully humidified atmosphere at 37 °C containing 5% CO_2, respectively. To demonstrate the localization of the drug-loaded nanoparticles in the cells, N-1-(3- triethoxysilypropyl) -N'-fluoresceylthiourea (FITC-APTES), prepared by stirring fluorescein isothiocyanate (FITC) in ethanol solution of APTES in the dark for 24 hours, was mixed with the MSN@cis-DDP@PAA nanocomposite to label FITC. Then the FITC-labeled nanoparticles loaded with cis-DDP (5 μg/ ml) were incubated with HeLa cells for 3 hours. The cells in the wells were washed three times with PBS and then visualized by fluorescence microscope after being stained with DAPI.

To measure the cellular uptake of the cis-DDP complexes, HeLa cells were seeded in 10 cm tissue culture dishes and incubated for 24 h. The medium was removed and replaced with fresh medium containing MSNs@cis-DDP@PAA/Ce6, after 3 h incubation, the cells were washed with PBS, trypsinized, and collected. The cells were counted and digested with HNO_3 (65%, 0.5 mL). The platinum content in cells was determined by ICP-MS. After analysis under confocal laser scanning fluorescence microscopy

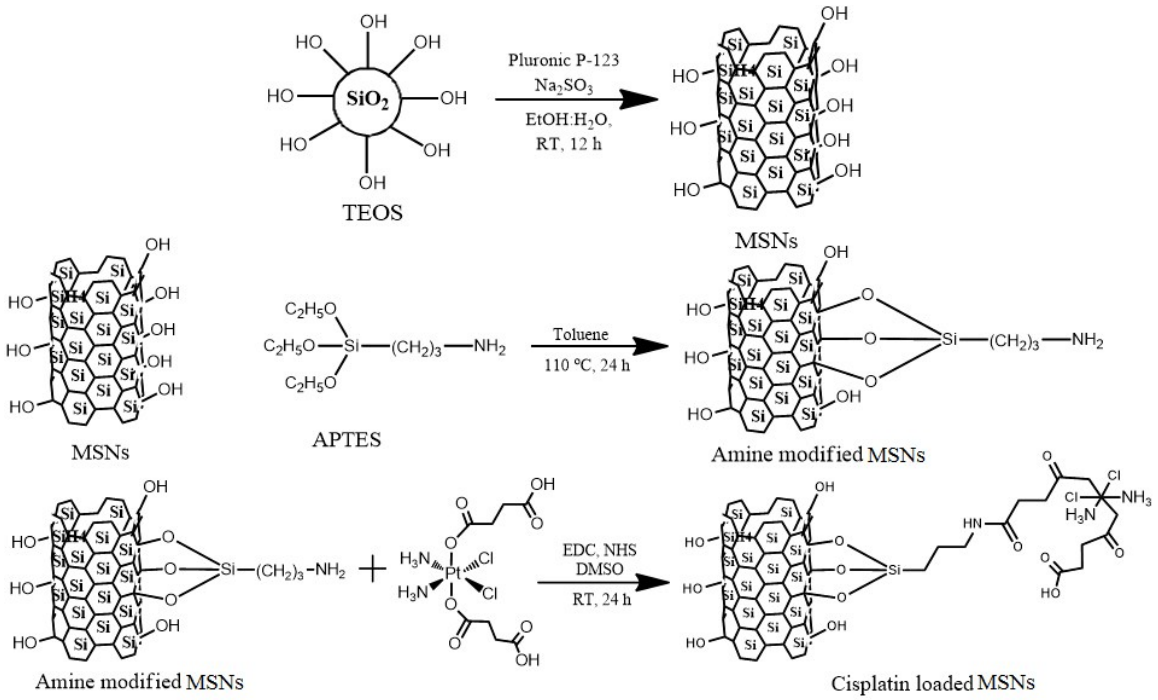
The mitochondrial membrane potential (ΔΨm), HeLa cells were dispensed at 8,000 cells/25 μl/well in MSN@cis-DDP/PAA. After the plates were incubated at 37° C overnight, the culture medium was removed from each well, and 25 μl of assay buffer containing different concentrations of FCCP, rotenone, or antimycin were added into each well. The plates were incubated at 37° C for 1 h, followed by the addition of 25 μl of dye solutions. The final concentrations of JC-1 10 μM and the plates were incubated at 37° C with the dyes for another 30 min and then washed with assay buffer. Fluorescence intensities 485nm excitations, 535nm emissions for JC-1 were measured using a fluorescence microscope.

Cytotoxicity of the prepared drug carrier nanocomposite

To determine cytotoxicity/viability, the HeLa and A549 cells were plated at a density of 1×10^4 cells/well in a 96-well plate at 37º C in 5% CO_2 atmosphere. After 24

hours of culture, the medium in the wells was replaced with the fresh medium containing the nanoparticles of varying concentrations. After 24 hours, 20µl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4) was added to each well. After 4 hours of incubation at 37° C in 5% CO_2 for exponentially growing cells and 15 min for steady-state confluent cells, the medium was removed. Formazan were solubilized with 150µl of DMSO and the solution was mixed under vigorous stirring to dissolve the reacted dye. The absorbance of each well was read on a microplate reader at 490 nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles was calculated by $[A_{test}]/[A_{control}] \times 100\%$.

Supporting Figures



Scheme S1. MSNs, cisplatin prodrug conjugated to the predominantly within the inner pore MSN surface and prodrug are electrostatically repulsive with affinity probe (PAA) were synthesized via a multistep procedure as depicted.

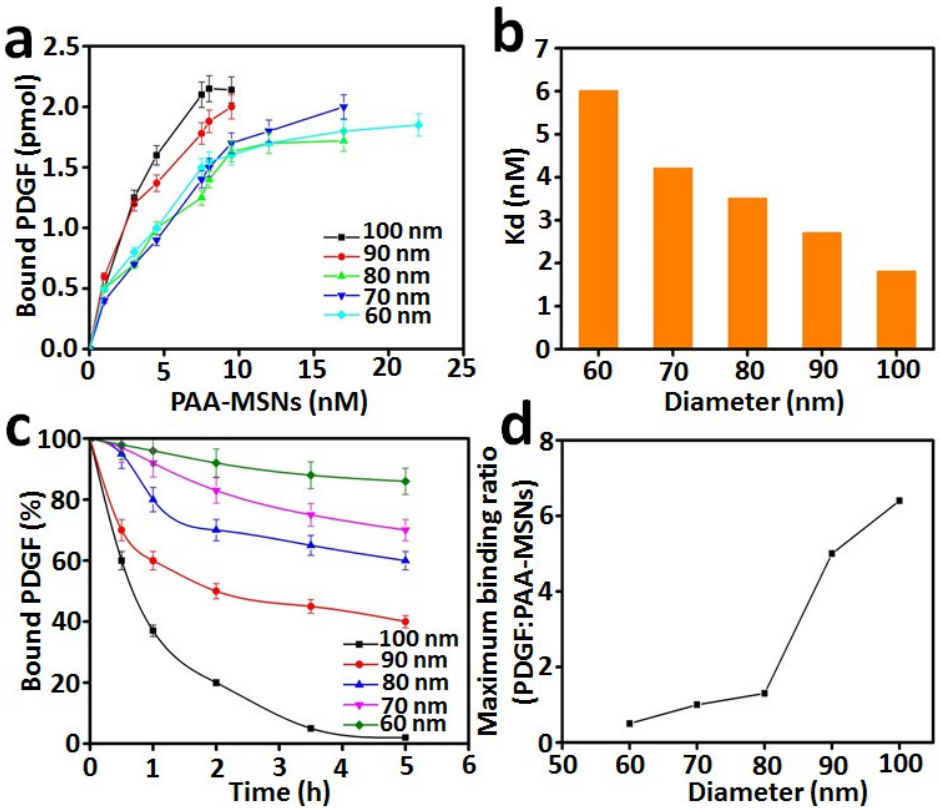


Figure S1. (a) Concentration-dependent binding of PDGF to different sized PAA-MSNs. Results are mean, n = 3. (b) Effect of nanoparticle diameter on the equilibrium binding constant (Kd) of PDGF. Kd values were estimated by fitting eq 1 to the data in a. (c) Dissociation of PDGF from PAA-MSN in the presence of excess unlabelled PDGF. On the basis of the law of mass action, an exponential curve was fitted to each data set and was used to estimate the dissociation rate constants for each nanoparticle. Results are mean, n = 3. (d) Effect of nanoparticle diameter on the number of PDGF molecules bound. Maximum binding ratio was determined from the data shown in a.

The PAA-MSNs bound to PDGF with saturating kinetics in Fig. 1a. The binding curves are shifted to the left with an increase in PAA-MSNs size indicative of increased binding affinity. To determine the binding kinetics for each of the nanoparticles, the Hill equation was fitted to the data by nonlinear least-squares regression. The dissociation constant Kd decreased progressively from approximately 6 nM for the smallest nanoparticles to less than 1.8 nM for the largest nanoparticles Fig. 1b. The change in free energy, estimated from the Gibbs equation, ranged from 48.8 to 52.5 kJ/mol, indicative of high affinity binding similar to that seen with antigen-antibody interactions. The decrease in Kd with

increasing diameter suggests an increase in the number of molecular interactions between the protein and the nanoparticles as surface area increased. Under these conditions of excess PDGF, no aggregation of the nanoparticles was observed. Fig. 1c illustrates the loss of surface-bound fluorescently labelled protein over 5 h. As the size of the nanoparticles increased, the rate of dissociation decreased, which further suggests greater molecular interaction between the PDGF with the larger nanoparticles. In the absence of excess protein, less desorption of PDGF from any of the nanoparticles was observed. This is most likely due to rapid reabsorption when no competing protein is present in the medium. The dissociation rate constant (koff) was estimated by fitting an exponential decay curve of the data. As the size of the PAA-MSNs increased, koff decreased from almost 1 h-1 to less than 0.1 h-1. The association rate constant (kon), calculated as the ratio of koff to Kd, was essentially independent of nanoparticle size, except for the 60 nm PAA-MSNs were kon was approximately 2-fold higher. With maximum binding capacity, the stoichiometry between the bound fibrinogen and the nanoparticles was dependent on particle size Fig. 1d. For the 60 nm PAA-MSNs, maximum binding occurred at approximately 1 PDGF per 2 nanoparticles, which is consistent with the multiple independent binding sites suggested by h = 1. For the 90 and 100 nm PAA-MSNs, maximum binding occurred at 1:1 stoichiometry. By contrast, PAA-MSNs larger than 90 nm bound multiple PDGF molecules, increasing linearly with the size of the nanoparticle. In our study, we investigated the HeLa cell (PDGF positive) and A545 (PDGF negative) cell uptake of PAA-MSNs by applying different sizes of nanoparticles and incubation times. Negatively charged PAA-MSNs were examined by the Hela cells with greater efficiency, most probably due to binding with the PDGF receptor. The positively charged PDGF receptor as expected gave the greatest uptake efficiency. The A549 showed the lowest level of uptake over the incubation periods. Interestingly, HeLa cell uptake for PAA-MSNs increased from approximately 2% efficiency after 30 min incubation to 8% after 2 h.

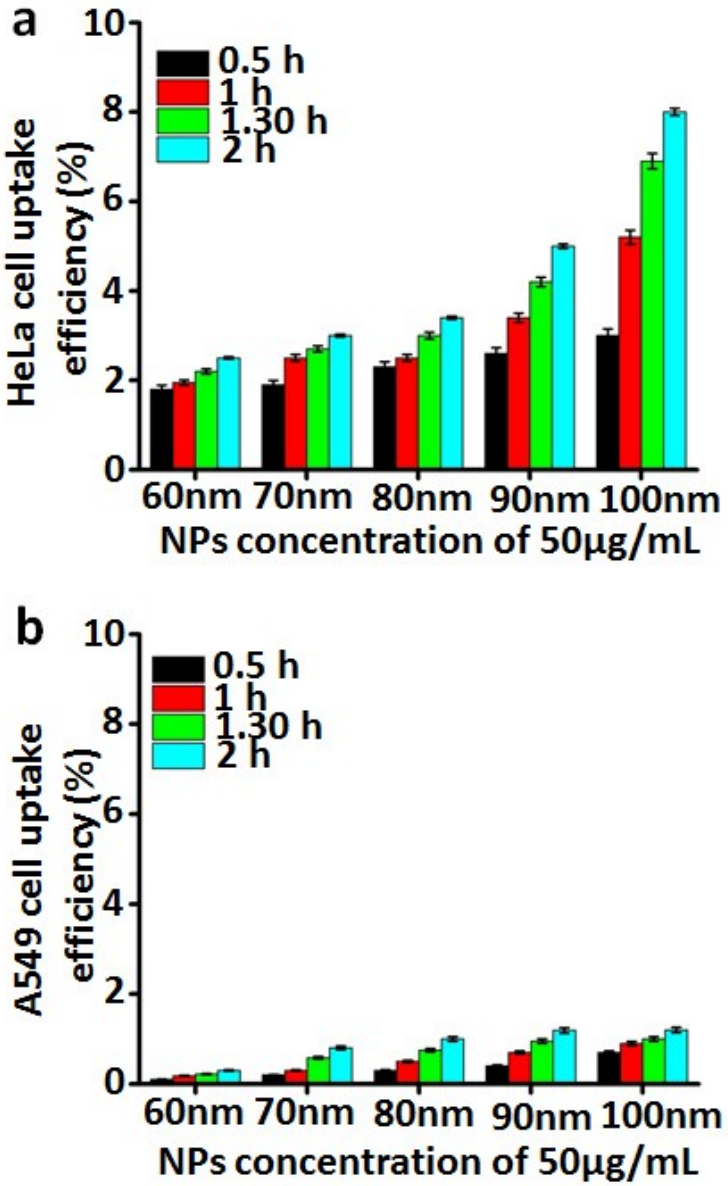


Figure S2. Cellular uptake efficiency of surface-modified PAA-MSNs by HeLa (PDGF +) and A549 (PDGF -) cells (small, 60 nm PAA-MSNs; medium, 80 nm PAA-MSNs; large, 100 nm PAA-MSNs).

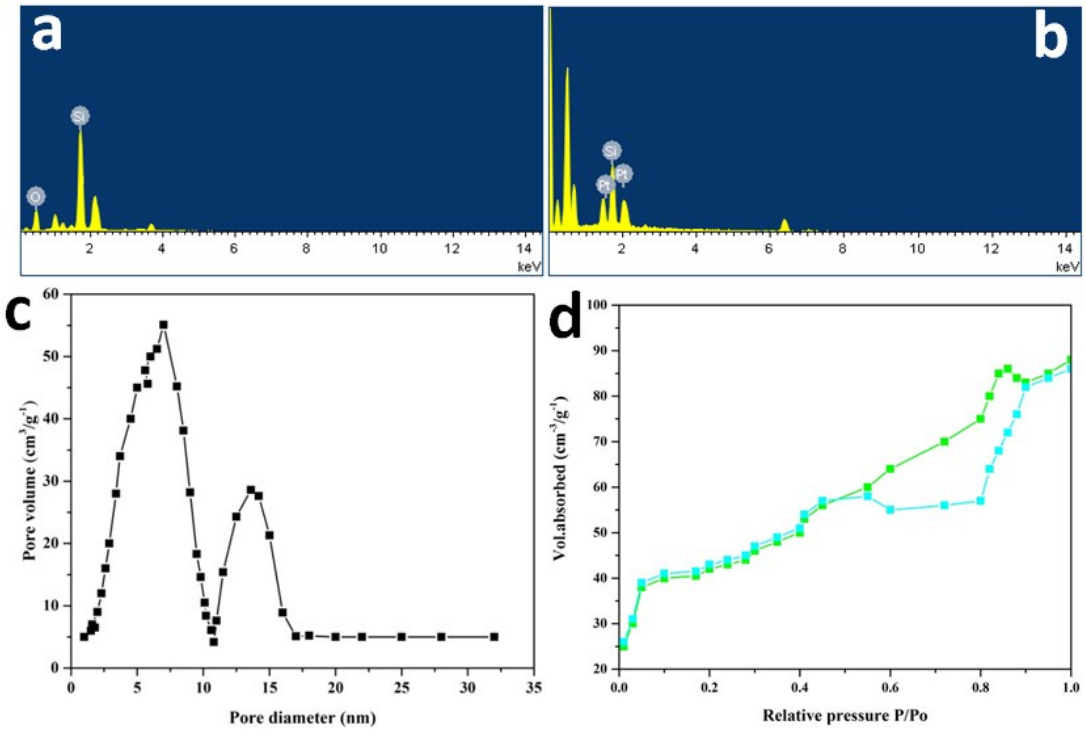


Figure S3. EDS spectrum of MSNs (a) after cis-DDP drug conjugation (b). Pore size distribution (c) and N2 adsorption-desorption isotherms (d)

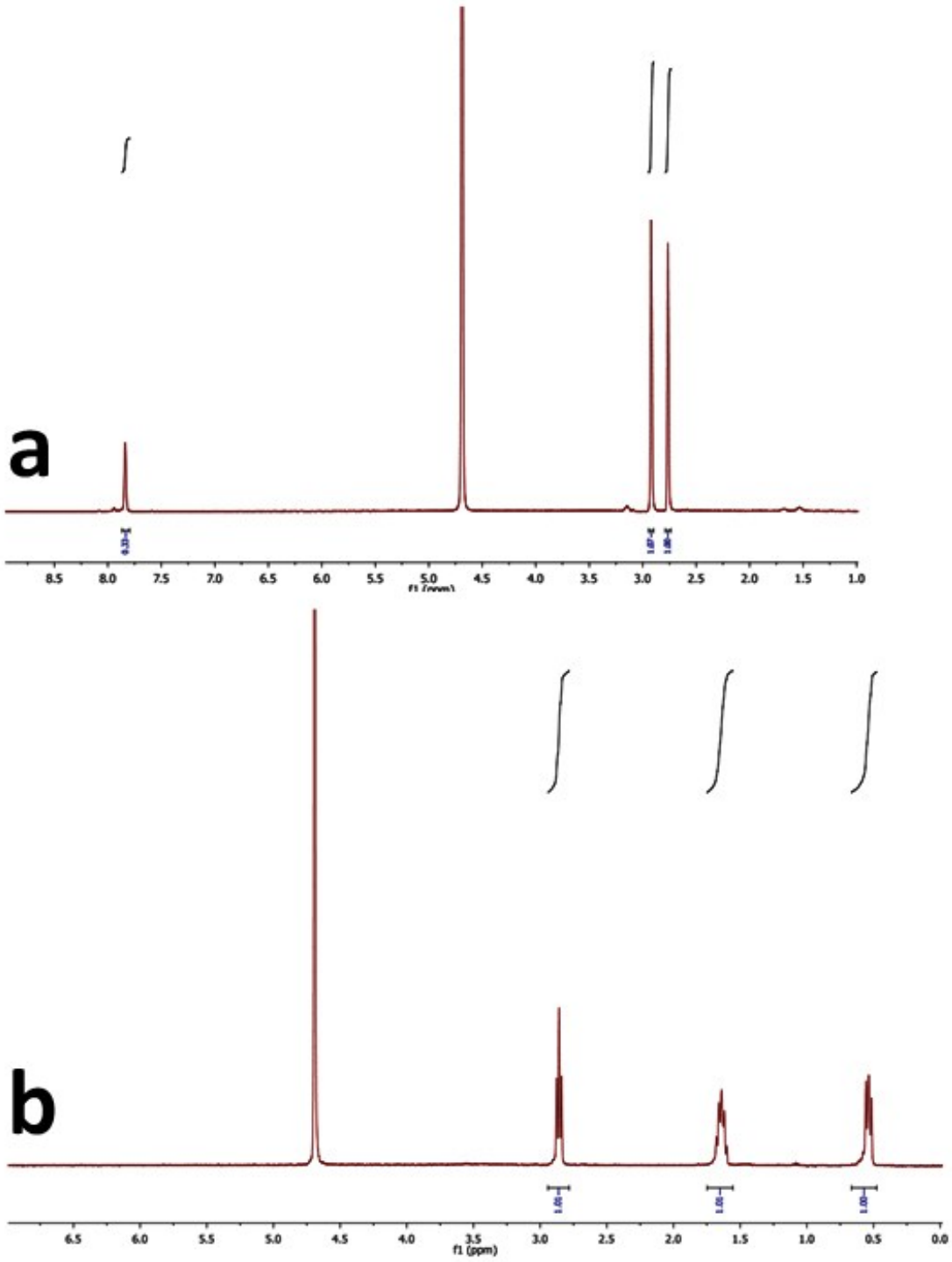


Figure S4. Proton NMR of the MSNs (a). After modification MSNs@cis-DDP@PAA (b)

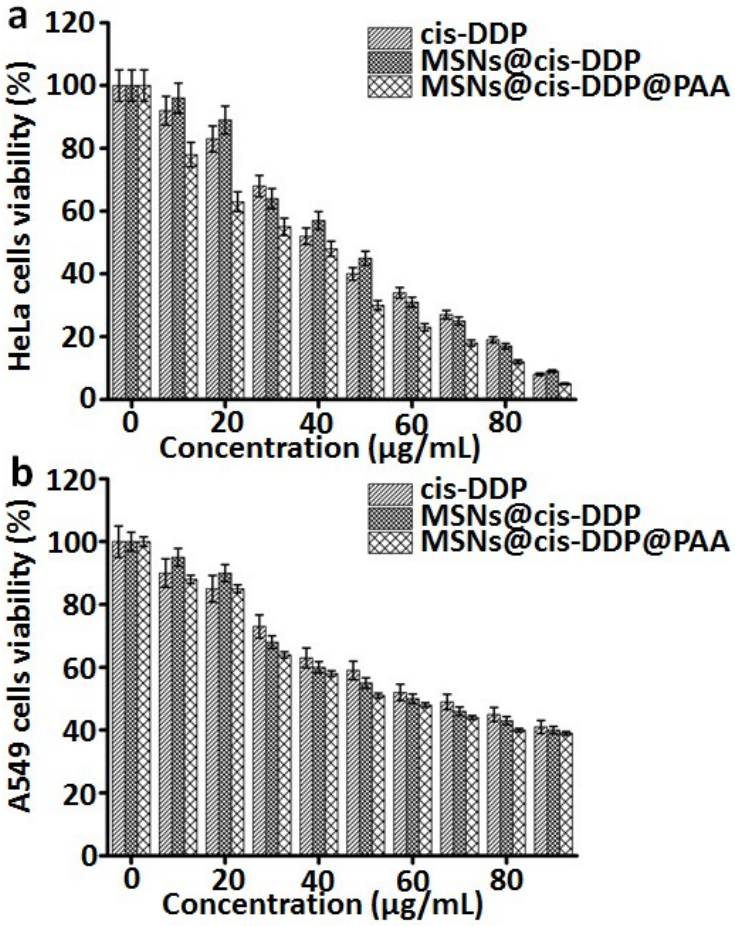


Figure S5. Anticancer effect of cis-DDP, MSNs@cis-DDP and MSNs@cis-DDP@PAA drug with various concentrations of HeLa and A549 cells.

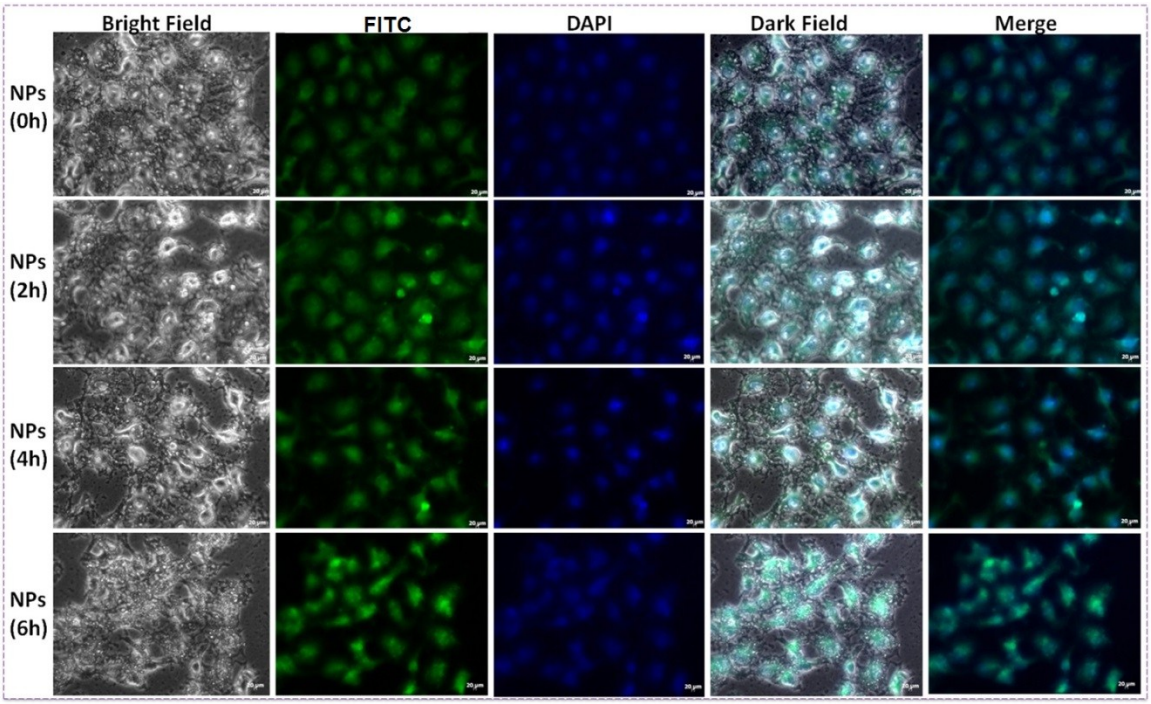


Figure S6. The fluorescence of A549 cells incubated with MSNs@cis-DDP@PAA (cis-DDP equivalent concentration is 0.5 μg mL^{-1}) for 0-6 h. FITC (green colour) and the nuclei stained with DAPI (blue colour) were recorded. The scale bar is 20 μm.

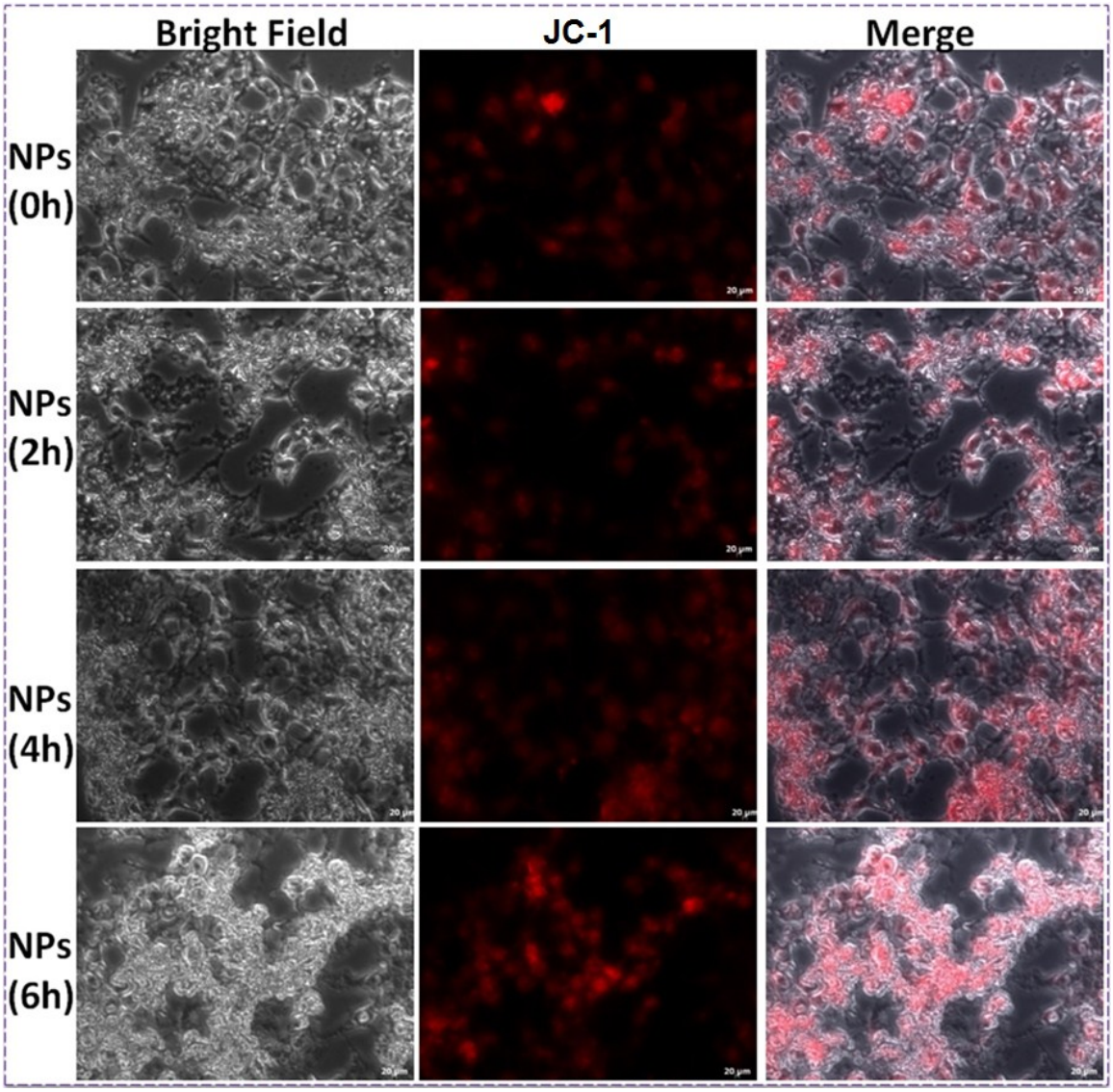


Figure S7. MSNs@cis-DDP@PAA nanocomposite incubate with HeLa cells at different hours (0-6) experiments that must be performed in parallel measuring Δψm with these cationic dyes to ensure appropriate interpretation of results. The scale bar is 20 μm

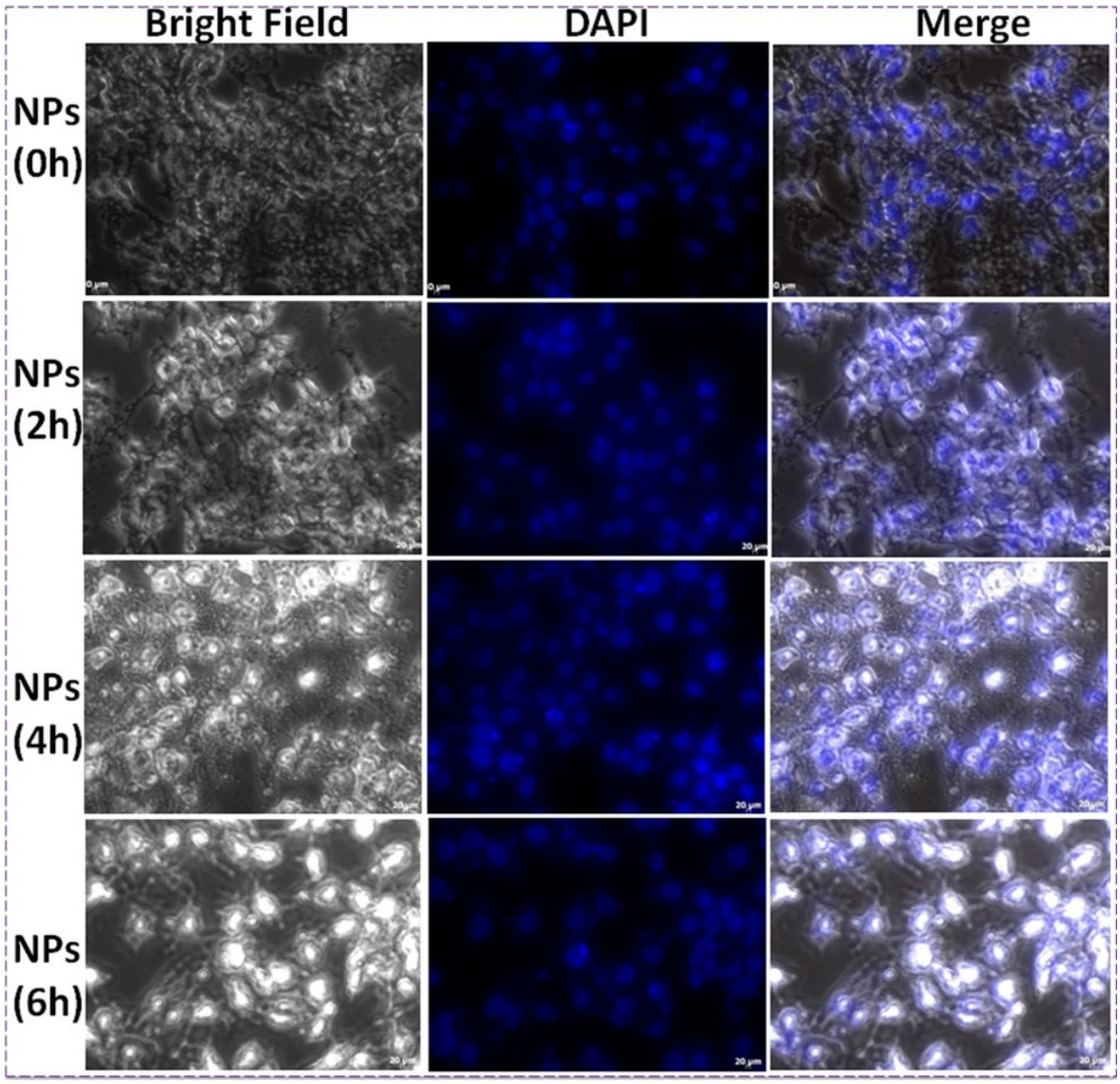


Figure S8. Micrograph of HeLa cells treated with MSNs@cis-DDP@PAA nanocomposite. HeLa cells were incubated with 5 μM and labeled with DAPI to show nuclear morphology. 0-6 hours incubated HeLa cells showing nuclear morphological changes. The scale bar is 20 μm

Reference

1. A. Byungjun, P. Juhee, S. Kaushik, P, Hansoo, J.K. Won, J. Mater. Chem. B, 2013, 1, 2829–2836.
2. W. Zhang, J. Shen, H. Su, G. Mu, J.H. Sun, C.P. Tan, X.J. Liang, L.N. Ji, Z.W. Mao, ACS Appl Mater Interfaces., 2016 ,8,13332-13340