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Supporting Information

One pot synthesis of doxorubicin loaded gold nanoparticles for sustained drug release

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Figures (S1-S12)

EXPERIMENTAL SECTION

Materials: All glassware were washed with aqua regia (3 HCl: 1 HNO_3), followed by rinsing several times with double distilled water. All the chemicals gold (III) chloride hydrate ($HAuCl_4$, 99.99%), doxorubicin hydrochloride, sodium citrate, were purchased from Sigma Aldrich. Sodium hydroxide pellet purified (NaOH, 97%), D-Tube Dialyzer (molecular weight cut off 12-14 kDa) were purchased from Merck. Double distilled deionized water with conductivity 18.3 mΩ (Elga Pure lab Ultra) was used throughout the preparation of solutions.

Synthesis of Dox-GNP conjugate : Synthesis of Dox-GNP conjugate was carried out in presence of O_2 under optimized conditions by adding 5 ml aqueous solution of $HAuCl_4$ (0.25 mM) to a 5 ml aqueous solution of Dox (10 μM) under moderate stirring for 2 min. Subsequently the pH of the reaction mixture is adjusted to 10 using 1 M NaOH. The mixture was incubated for 3 h at 50 °C under moderate stirring. The Dox-GNP conjugate formation was indicated by the change in the color of the solution from pale yellow to purple. For synthesis of Dox-GNP conjugate in anaerobic condition, the aqueous solution of $HAuCl_4$ (0.25 mM) and Dox (10 μM) was purged with N_2 gas for 10 minute and then the reaction was continued under similar condition as earlier but for 24 h. Synthesis of Dox-GNP conjugate nanoparticles were also carried out with N-Ethyl derivative of Dox under similar aerobic condition.

Synthesis of N-Ethyl derivative of Dox: To the dichloromethane (DCM) solution of Dox (10 μM) triethylamine (20 μl) at 0°C was added and reaction was stirred at this temperature for 10 minute followed by the addition of ethyl bromide (6 μl).[1] The reaction mixture was stirred at room temperature for 6 h. After that reaction mixture was concentrated under vacuum to obtained the –$NH(C_2H_5)$ derivative of Dox and used for further reaction.

Characterizations of Dox-GNP conjugate:

The particle size and dispersity of the synthesized nanoparticles were analyzed using a TECNAI 200 kV (Fei, Electron Optics) transmission electron microscope (TEM) with 200kV input voltage. The hydrodynamic diameter (D_H) of the nanoparticles were measured by dynamic light scattering (DLS) measurements using zeta-sizer-nano(Model: equipped with a He-Ne laser illumination at 633 nm and a single photon counting avalanche photodiode for signal detection, Malvern Instrument). UV-vis absorbance spectra of the Dox-GNP conjugate were measured by UV-2576 series from Schimadzu. Steady-state fluorescence data were recorded in a Horiba Fluorolog-3 spectrofluorometer and Thermo scientific Nano-Drop 3300 fluorospectrometer. The fluorescence spectra were measured with a 10 mm path length quartz cuvette. Both the emission and the excitation slits were kept at 3 nm. FTIR spectra were recorded using Agilent Spectrometer (Carry 660spectrometer).Circular dichroism (CD) spectra were measured using a Jasco-1500 spectrophotometer equipped with a thermostatically controlled cell holder. High resolution mass spectra of pure Dox and Dox released from Dox-GNP conjugate was recorded on HRMS Bruker impact HD. The protein concentration used for protein conformational studies is10 µM for all the measurements. The final spectra were obtained by subtracting the buffer (PBS pH 7.4) contribution from the original sample spectra. Optical density of the sample (MTT assay) was recorded using multimode plate reader (TECAN infinite M200 PRO).

Cell culture:

A human breast cancer cell line MDA-MB 231, was obtained from NCCS (national centre for cell science), Pune, India. All cell culture related reagents, such as DMEM (dulbecco's modified eagle medium), FBS (fetal bovine serum), penicillin, MTT (methyl thiazolyltetrazolium) were purchased from Sigma Aldrich. MDA-MB-231 cells were grown in normal DMEM culture medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Cytotoxicity assay:

The in-vitro cytotoxicity was measured using a standard MTT assay. MDA-MB 231 cells were seeded into 96-well cell culture plate at 10^4/well and then incubated for 24 h at 37°C under 5% CO_2. The cells were incubated with varying concentration of free Dox and Dox-GNP conjugate for 72 h and 5 µM concentrations of free Dox and Dox-GNP conjugate for 24 h to 72 h, followed by 1 h incubation with the MTT reagent. Then the optical density of the sample was recorded using multimode plate reader (TECAN). The IC_{50} value for pure Dox and Dox-GNP conjugate was determined against the same cell line in dose dependent manner. The concentration of Dox and Dox-GNP conjugate was varied from 2 µM to 20 µM.

In-vitro drug release:

The in-vitro release of Dox from Dox-GNP conjugate nanoparticles was performed at 37 °C and pH 6.6 and 7.4 in PBS buffer. 2 ml of Dox-GNP conjugate dispersion was transferred to the dialysis tube, which was then immersed into 40 ml of PBS buffer at 37°C. At time interval of 1 h, 1 ml of release media was taken out for absorbance measurement and replenished with an equal volume of the same media. The amount of Dox released was determined by absorbance measurement at 480 nm. The same sample was utilized for fluorescence recovery experiment. Fluorescence measurement (λ_{em} = 594 nm) using Thermo scientific Nano-Drop 3300 fluorospectrometer, after 80 h fluorescence spectra was measured using Horiba Fluorolog-3 spectrofluorometer.

For performing the drug release experiment in the presence of HSA protein, Dox-GNP conjugate (2ml) was incubated with HSA protein (200 µl of 100 µM) for 2 h. After incubation, HSA coated Dox-GNP conjugate was centrifuged for 10 minute at 15000 rpm to remove unbound HSA. This obtained pellet was redispersed in 2 ml of distilled water. The time dependent release studies of Dox from Dox-GNP-HSA were carried out as explained above.

Cell Uptake: MDA-MB 231 cells were seeded onto labtech culture plates and incubated at 37 °C in a 5% CO2 humidified atmosphere for 24h. The Dox-GNP conjugate nanoparticle was added to the cells followed by further incubation at 37 °C for 0 h, 12 h, 24 h, 48 h, and 72 h. The medium was removed and the cells were washed two times with PBS buffer. The cells were further incubated at 37 °C for a certain period of time, and then the images of cells were recorded by Nikon confocal microscope (Nikon Ti). Fluorescence image of cell was recorded using a 480 nm excitation source and corresponding filter.

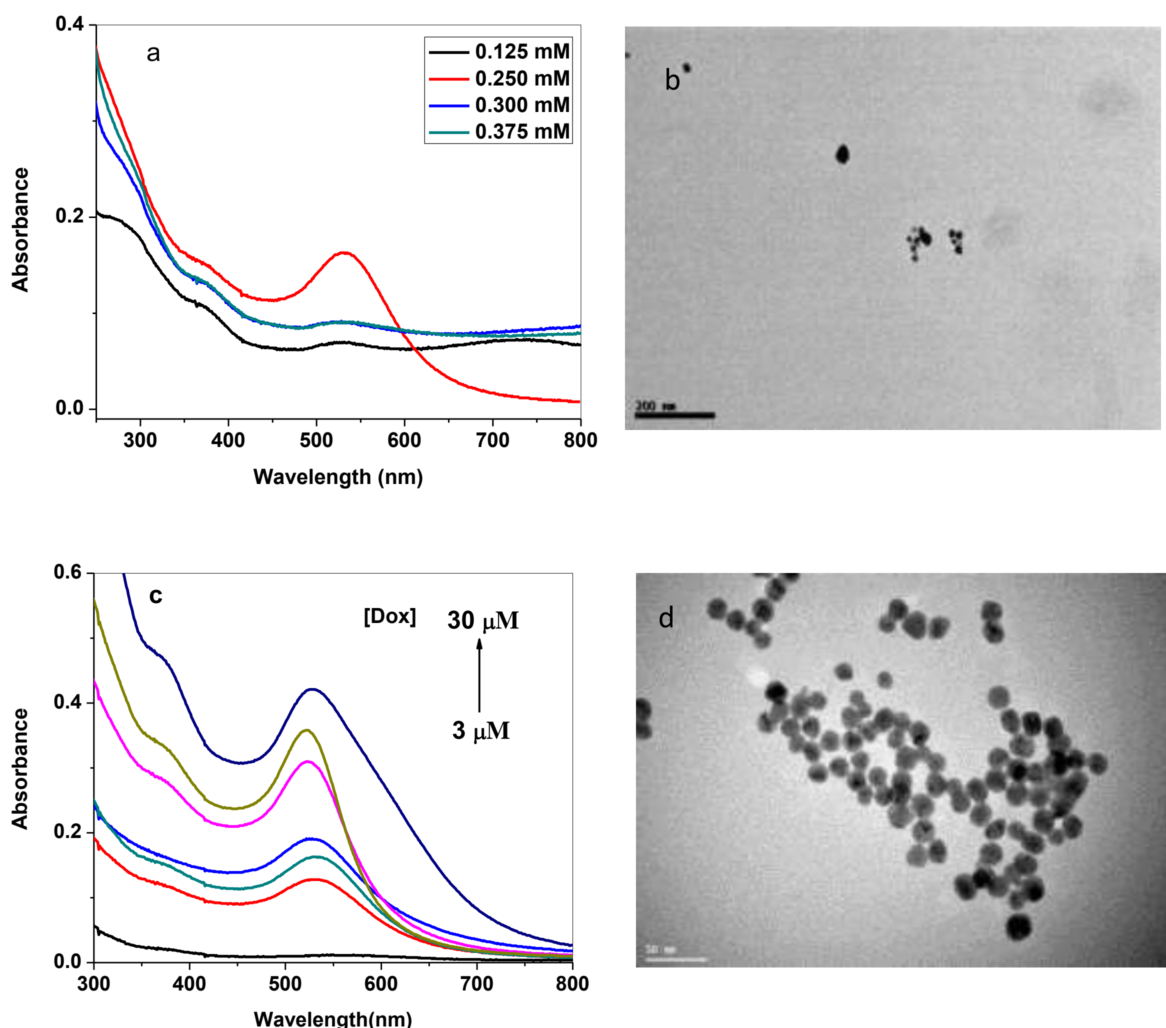

Figure S1: Synthesis of Dox-GNP conjugates (a) UV-vis absorption spectra at different concentration of $HAuCl_4$ (b) TEM image at 0.125 mM HAuCl4 in the presence of 10 µM Dox, TEM image confirm that the lower concentration of $HAuCl_4$ is not enough for nanoparticle formation (c) synthesis of Dox-GNP conjugate at different concentration of Dox in the presence of 0.25 mM $HAuCl_4$ at 50°C and pH 10 under aerobic condition (d) TEM image of Dox-GNP conjugate at 30 µM Dox concentration. The concentration of Dox above 10 µM showed possible agglomeration. Moreover, at higher concentration of Dox (> 15 µM) the shoulder at 360 nm (n-π* transition of C=O of Dox) indicates the presence of unreacted Dox molecule at concentration (> 10 µM).

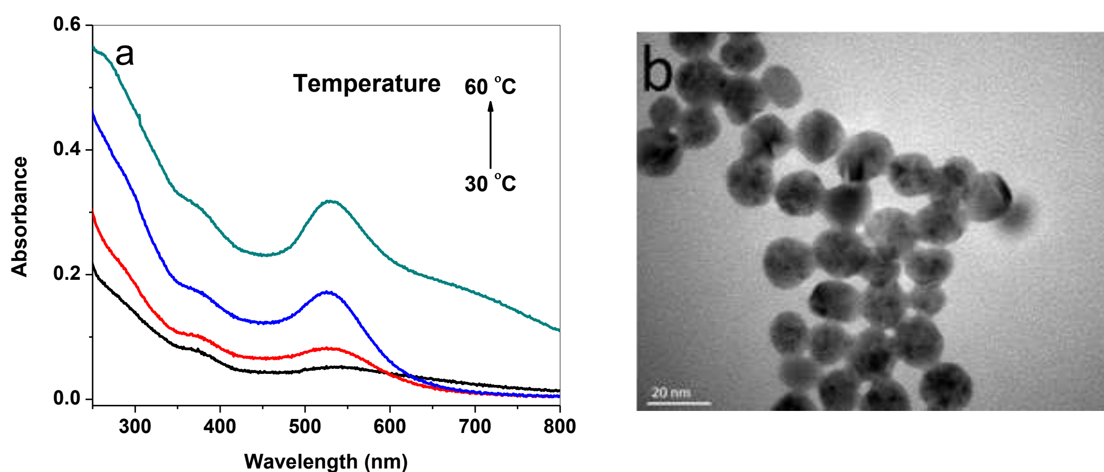

Figure S2: Synthesis of Dox-GNP conjugates (a) at different reaction temperatures in presence of 0.25 mM $HAuCl_4$ and 10 µM Dox at pH 10. (b) TEM image of Dox-GNP conjugate at 60 °C. It is observed that when the reaction is carried out at 30 and 40 °C, the extant of Dox-GNP conjugate formation is very less, and at 60 °C a shoulder around 685 nm is observed in addition to characteristic SPR band at 525 nm, suggesting probable agglomeration. Therefore, 50 °C was chosen as the optimum reaction temperature for the synthesis of Dox-GNP conjugate.

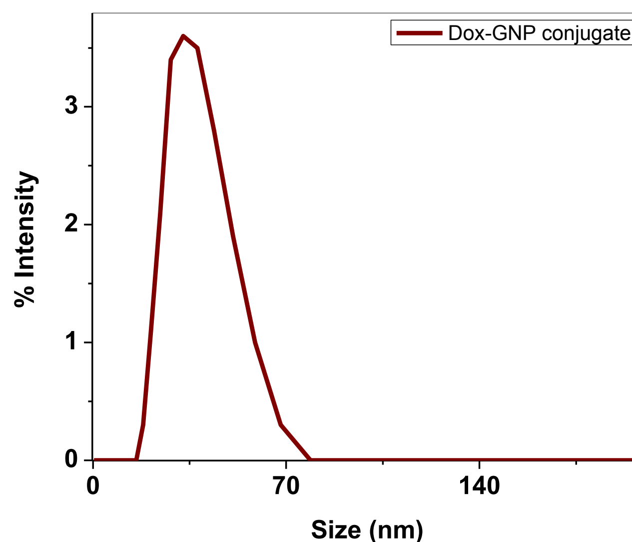

Figure S3: Hydrodynamic size of the synthesized Dox-GNP conjugate. The hydrodynamic size of the nanoparticles is usually bigger than the actual size, which explains the larger size of the GNP (~30 nm) obtained from DLS measurement.

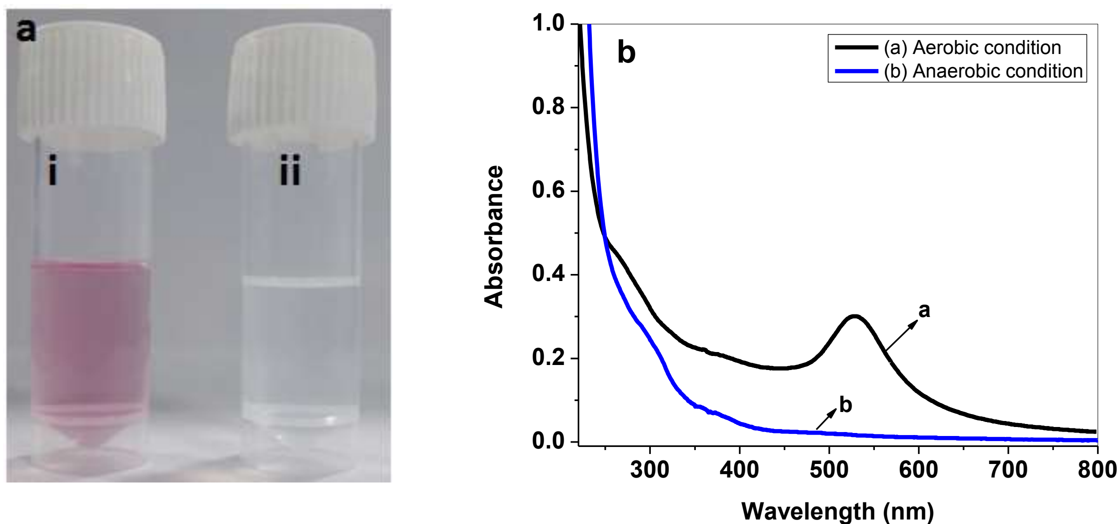

Figure S4:(a) Color image of as-synthesized Dox-GNP conjugate (i) in aerobic condition (in the presence of molecular O_2) and (ii) Anaerobic condition (in the presence of N_2), and, (b) UV-vis absorption spectra of as-synthesized Dox-GNP conjugate in (curve a) aerobic condition, and (curve b) in anaerobic condition.

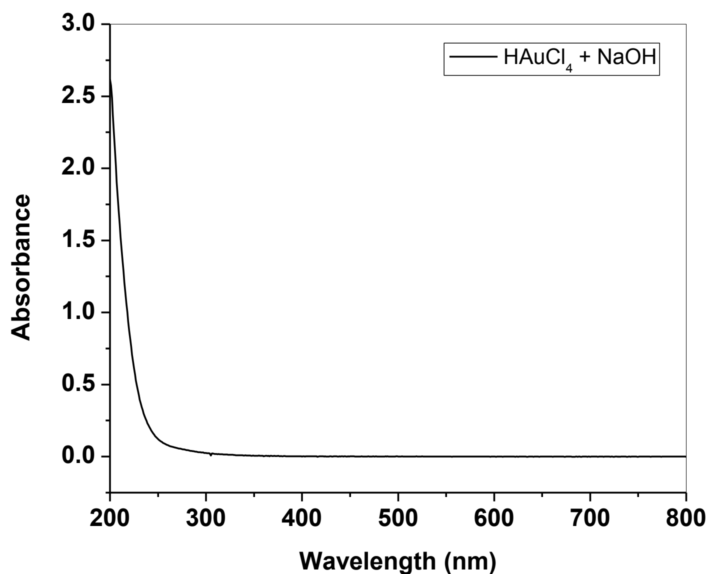

Figure S5: UV-vis absorption spectra of the sample prepared in the absence of Dox keeping all other synthesis condition same, [$HAuCl_4$] = 0.25 mM, at 50°C and pH 10. Under basic condition, $AuCl_4^-$ gets converted into a more hydroxylated species which is confirmed by the absence of any peak in the spectra.[2]

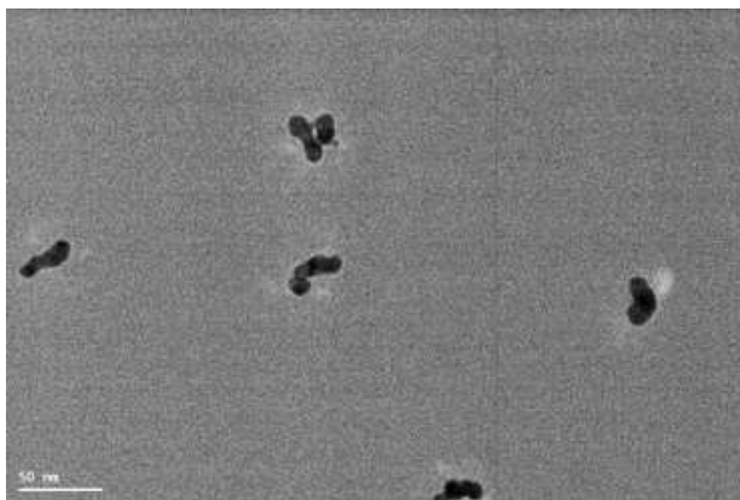

Figure S6: TEM image of the nanoparticles synthesized in the presence of N-ethyl derivative of Dox, [$HAuCl_4$] = 0.25 mM, at 50°C and pH 10. Particle formation is clearly visible from the image.

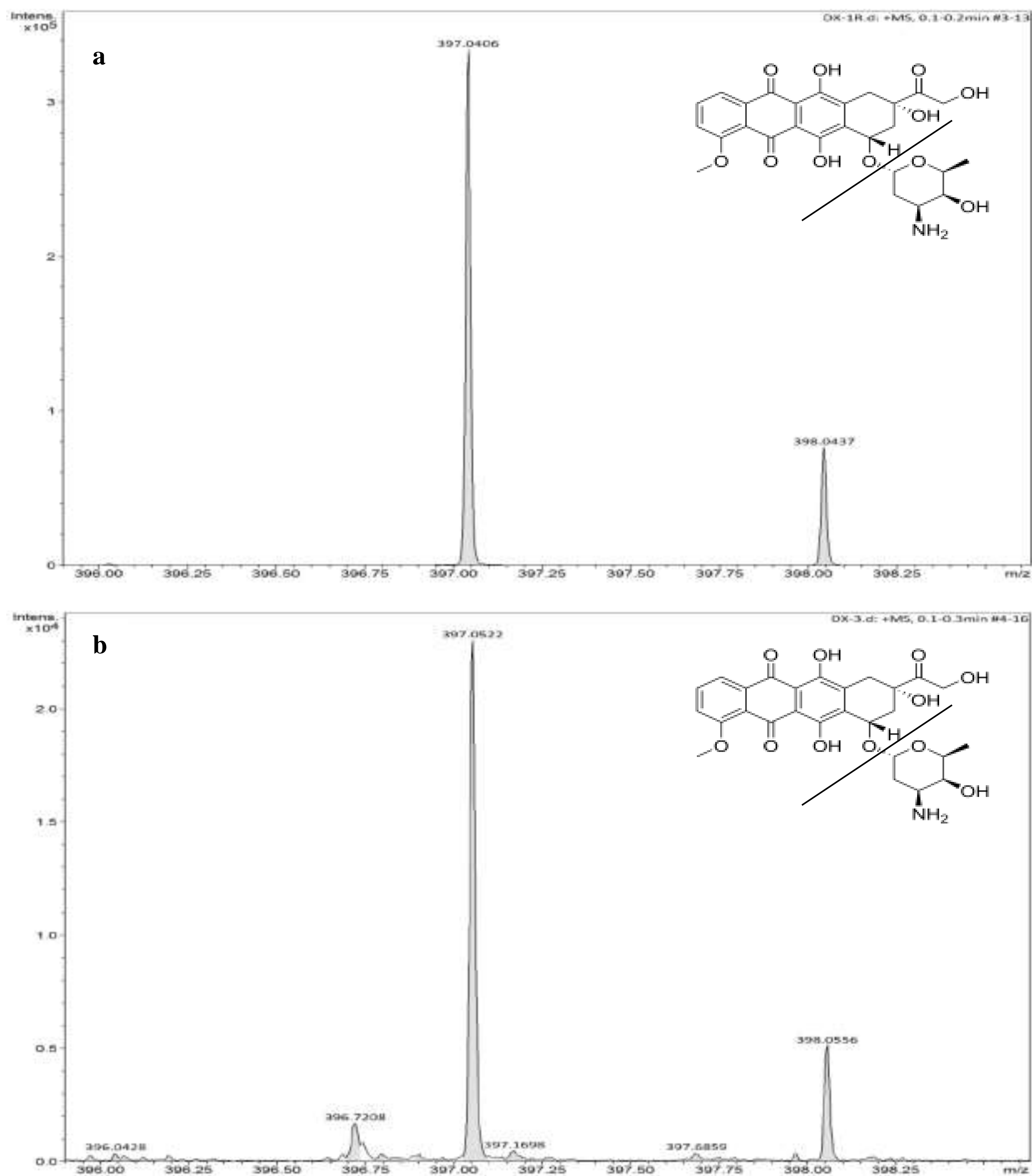

Figure S7: Mass spectra of (a) pure Dox (b) Dox released from Dox-GNP conjugate showed mass peak at 397 corresponding to anthraquinone ring of Dox.[3] The good match in the mass peak corresponding to the anthraquinone ring confirms that "effective part" of the drug remains unchanged during the synthesis.[4]

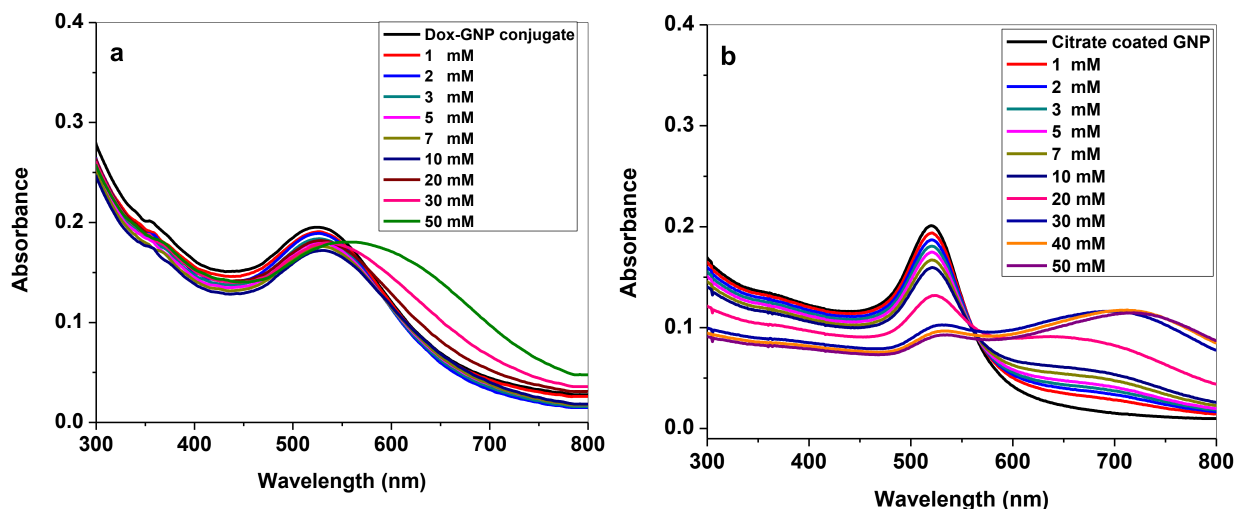

Figure S8: Effect of NaCl on stability of (a) Dox-GNP conjugate. The synthesized system is stable upto 20 mM of NaCl concentration. (b) Effect of NaCl concentration on citrate coated GNP. The data showed that in this case a new band in the red region of the spectrum starts appearing even in the presence of 2 mM of NaCl concentration.

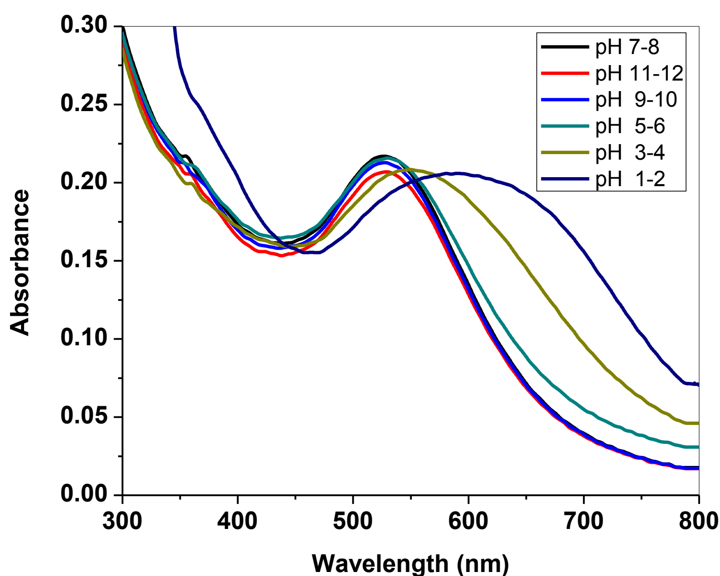

Figure S9: Effect of pH on the stability of Dox-GNP conjugate nanoparticles. It is observed that Dox-GNP conjugate is quite stable in the pH range of 5 to 12 but at lower pH (pH< 4) the nanoparticles tend to lose their stability.

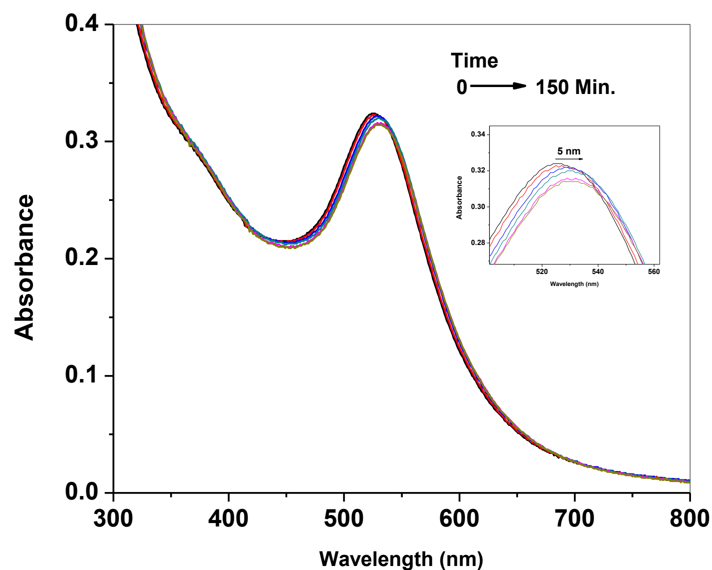

Figure S10: Time dependent kinetics of the protein corona formation around Dox-GNP conjugate nanoparticles. A red shift of 5 nm (inset) in the SPR band of the Dox-GNP conjugates upon equilibration for 2 h with HSA protein (10 µM), confirm the monolayer formation around Dox-GNP conjugate.

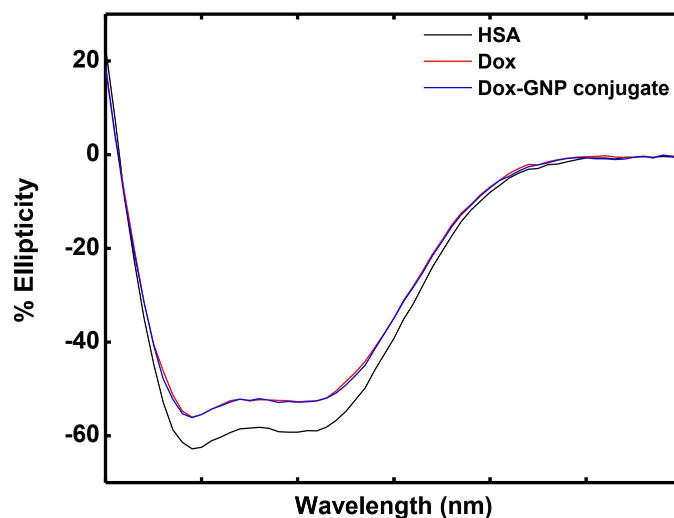

Figure S11: Secondary structure changes of HSA protein in the presence of Dox and Dox-GNP conjugate. The secondary structure of HSA protein was found to be unperturbed by Dox-GNP conjugate.

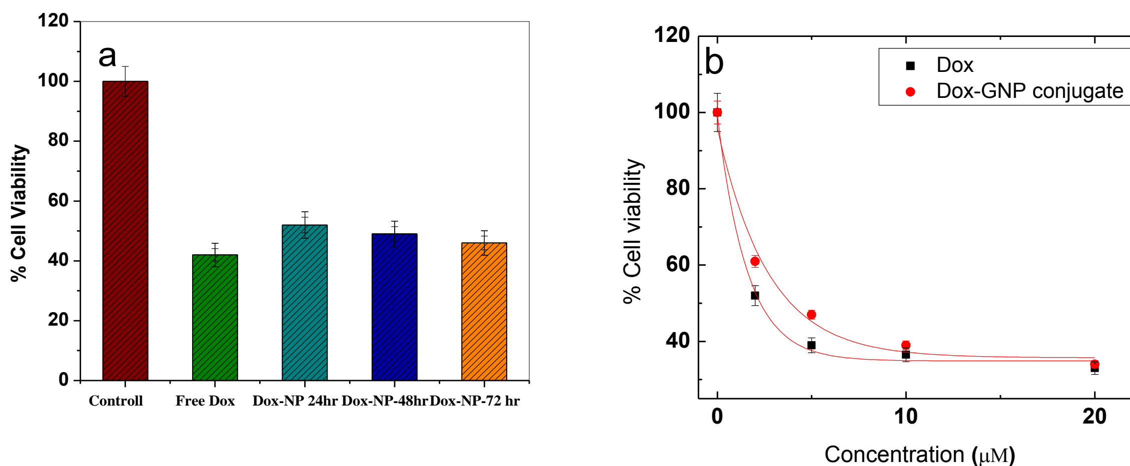

Figure S12: (a) Cytotoxicity study of free Dox and Dox-GNP conjugate at different time interval using MTT assay. (b)) Dose dependent curve of MDA-MB-231 cells after treatment with free Dox and Dox-GNP conjugate at varying concentration, Dox and Dox-GNP conjugate were incubated with cells for 72 h at 37°C.

References:

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