Supporting Information

Cell Deformation and Acquired Drug Resistance: Accounts of Major Influence of Drug-nanocarrier Delivery Systems

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1. Synthesis of nanocarriers

Materials. Doxorubicin hydrochloride (Dox), phosphate buffered saline (PBS), Trypsin EDTA, antibiotic-antimycotic solution, dimethyl sulfoxide anhydrous (DMSO), thiazolyl blue tetrazolium bromide (MTT), 4'-6-diamidino-2-phenylindoledihydrochloride (DAPI) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl) were obtained from Sigma Aldrich. Graphene grade 2 (G) was purchased from Global Nanotech. L-cysteine hydrochloride monohydrate (Cys), Dulbecco's Modified Eagle Medium, high glucose (DMEM) and fetal bovine serum, heat inactivated (FBS) were procured from Himedia. 4-dimethylaminopyridine, 98% (DMAP) was obtained from Avra. Ultrapure (Type 1) water produced from Direct Q-3 system of Millipore was used throughout. HeLa cancer cells were received from National Centre for Cell Science, Pune, India.

Preparation of Fe_3O_4 NPs. Fe_3O_4 was synthesised using ferrous and ferric salts as reported earlier[1]. The salts were precipitated using 25% ammonium hydroxide. The solution containing the precipitate was then heated to 80 °C for 30 minutes. Fe_3O_4 NPs were isolated by magnetic separation. The particle

Synthesis of Cys-Fe_3O_4. Fe_3O_4 particles (5 mg) were suspended in a mixture of ultrapure water (0.15 mL) and methanol (0.05 mL). The dispersion was sonicated for 15 min. Cys (4 mg) was dissolved in ultrapure water (800 mL) and then added to the suspended Fe_3O_4 NP solution (pH

4.0). The suspension was re-sonicated for 2 h. The Cys-Fe_3O_4 complex was subsequently isolated by magnetic separation to remove unreacted Cys. The conjugate obtained was washed with repeated chemo-cycles of ultrapure water. Finally, conjugates dried under vacuum at RT.

Preparation of G-COOH. 200 mg of G was continuously stirred with 5 mL of H_2SO_4 (98%) for 2 h in ice-bath maintained between 0-5 °C. 600 mg $KMnO_4$ was added slowly, at reaction temperature below 20 °C. The reaction mixture was further stirred at 35 °C for 2 days. After adding 10 mL of water, brown coloured solution was obtained. The solution was subsequently heated to 98 °C for 15 min. Additional 20 mL water was added and stirred continuously. Finally, the solution was treated with 1 mL H_2O_2 and the product obtained was light brown in colour. The G oxide (G-COOH) prepared was washed repeatedly with HCl and DI water[3]. The amount of carboxylic groups generated on G was determined by acid base titration.

Synthesis of G-Dox. 5 mg of G-COOH conjugate was added to 1 mg of Dox dissolved in ultrapure water (1 mL) in the presence of EDC.HCl and DMAP. The reaction was stirred for 24 h at RT (pH 4.0). The modified G-COOH was collected and washed with ultrapure water by centrifugation to remove unconjugated Dox and dried at RT to obtain G-Dox.

Synthesis of G-Cys-Fe_3O_4. 10 mg of G-COOH was added to 3 mg of Cys-Fe_3O_4 complex suspended in ultrapure water (1 mL) in the presence of EDC.HCl and DMAP. The reaction was continuously stirred at RT for 24 h (pH 4.0). The conjugated G-Cys-Fe_3O_4 was washed with ultrapure water using centrifugation to remove unbound components and dried at RT to obtain G-Cys-Fe_3O_4 conjugate.

Synthesis of G-Cys-Fe_3O_4-Dox: 5 mg of G-Cys-Fe_3O_4 conjugate was added to 1 mg of Dox initially dissolved in ultrapure water (1 mL) in the presence of EDC.HCl and DMAP. The reaction was stirred at RT for 24 h (pH 4.0). The modified G was collected and washed with ultrapure water by ultracentrifugation to remove unreacted Dox, then dried at RT to obtain G-Cys-Fe_3O_4-Dox.

2. Characterization

The infrared absorption spectra of the synthesized carbon nanocarriers were obtained using FTIR 4600, JASCO in the range between 4000 cm^{-1} and 400 cm^{-1}. Fluorescence spectrophotometer (Cary 100; Varian) was used to measure fluorescence emission spectra at different time intervals and different concentration. Prior to recording the spectra, the samples were excited at 480 nm and then scanned from 490 nm to 800 nm. The temperature was maintained at 25 °C throughout the study. DLS was used for measuring the hydrodynamic particle size by using Malvern Mastersizer 2000. Transmission electron microscopy (TEM) imaging was carried out using FEI Technai G2 20-S Twin operated at 200 kV. The sample was dispersed in water with sonication for 15 min. A drop of sample was placed on the copper grid and dried at RT and placed in the microscope.

***In vitro* release studies:** The synthesized multicomponent conjugates were stirred at 100 rpm in two different solutions maintained at two pH conditions (pH 7.4 and pH 5.0) for defined time intervals (1, 4, 8, 24, 48 h), centrifuged and 100 μL of clear aliquots of each were taken to estimate the amount of Dox released by HPLC method. The chromatographic conditions required a stationary phase of octadecylsilyl silica gel (5 μm). The mobile phase comprised of a mixture of equal volumes of acetonitrile and a solution containing 2.88 g.L-1 of sodium dodecyl sulphate and 2.25 $g.L^{-1}$ of phosphoric acid with a flow rate of 1 $mL.min^{-1}$. The wavelength for detection was set at 254 nm. The chromatogram of the aliquots was compared with a standard curve to obtain the amount of drug released.

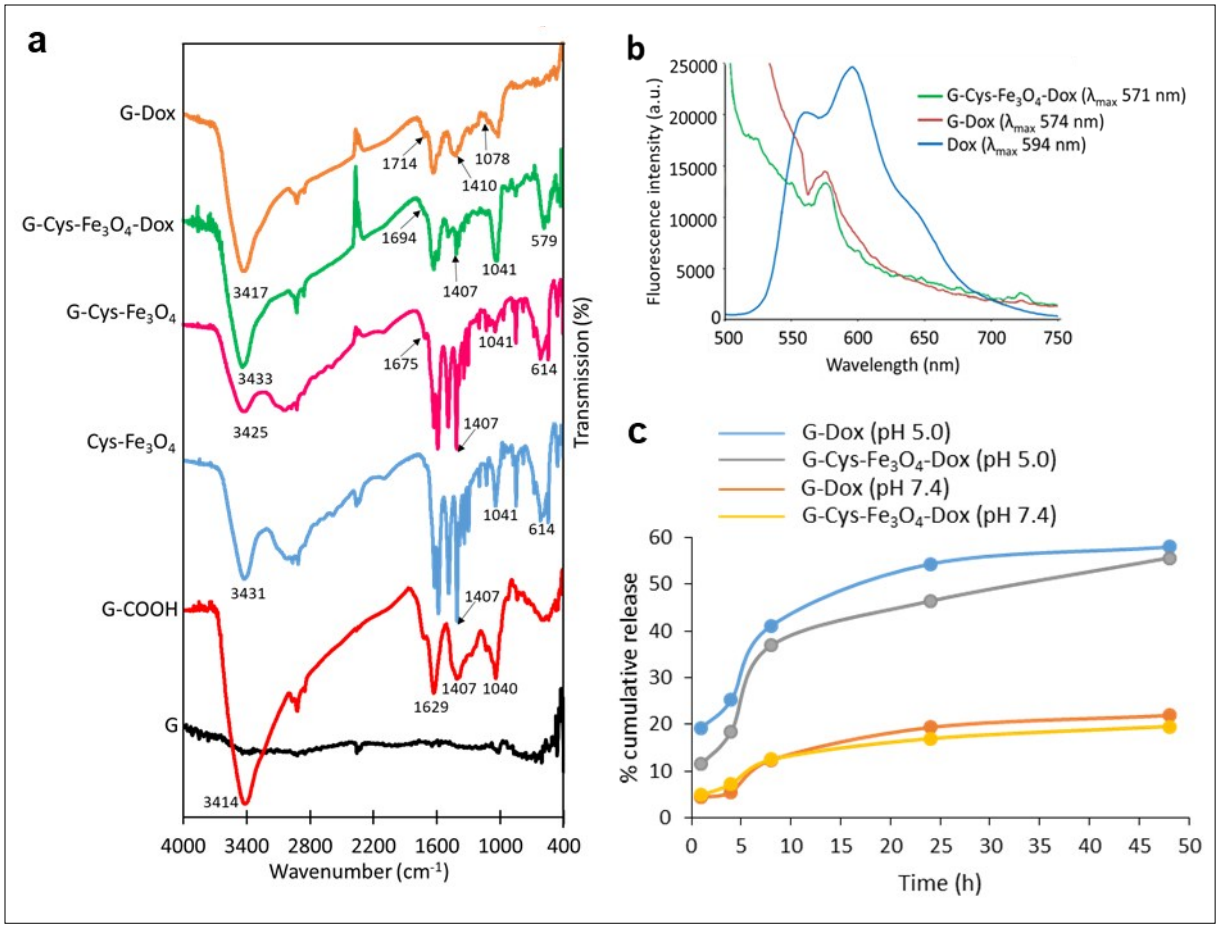

Figure S1. Characterisation of the synthesised Dox nanocarriers. (a) FTIR spectra for G, G-COOH, Cys-Fe_3O_4, G-Cys-Fe_3O_4, G-Cys-Fe_3O_4-Dox, and G-Dox. (**b**) Fluorescence emission spectra of Dox and Dox nanocarriers, λ_{ex} was set at 480 nm and pH of buffer used was 7.4. (**c**) *In vitro* drug release profile of Dox from the synthesized nanocarriers at pH 5.0 and pH 7.4. Sampling time-points were 1 h, 4 h, 8 h, 24 h and 48 h.

3. Cell viability studies

The cytotoxic activity of Dox was quantitatively determined by MTT assay. Briefly, 5000 cells/well were seeded in a 96-well plate and maintained for 24 h at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS. Free Dox was added in varying concentrations (0.1–0.3 μM) and incubated for 48 h, following which MTT reagent was added. Cells were washed with PBS and DMSO was added (100 μL) to each well. The cells

dissolved the precipitate following which the absorbance was measured at 570 nm using a Tecan Plate Reader. Background readings (blank) were obtained from cell-free wells containing only DMSO. Percentage cell viability was calculated as:

$(A \times 100)/C$

where, A = test sample MTT absorbance,

C = control MTT fluorescence.

G conjugates were weighed, according to their Dox content, equivalent to the IC_{50} concentration of free Dox and cell viability was determined similar to the procedure mentioned above.

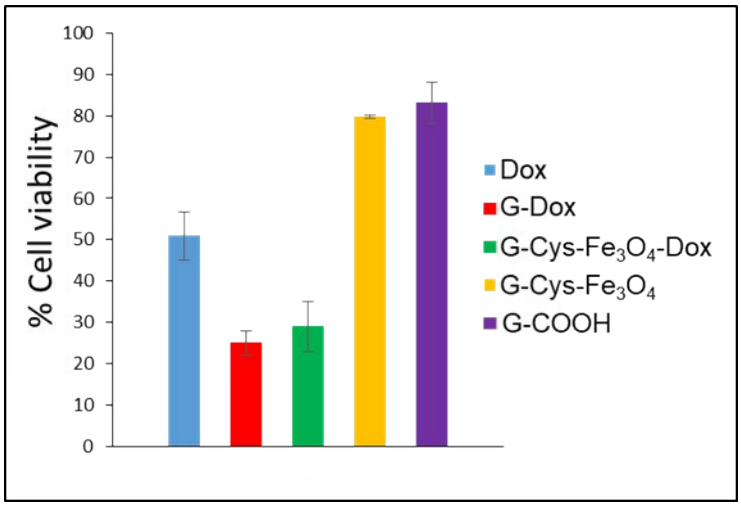

Figure S2. Cell viability studies. The comparative cell viability evaluation of HeLa cells treated for free Dox and Dox nanocarriers were performed as per the MTT assay protocol. Concentration of Dox used was 0.26 μM (IC_{50} value).

4. Fluorescence microscopy

The microscopic images were captured using a fluorescence microscope (Axio Observer.A1, Zeiss, India) as well as confocal microscopy (Confocal Leica TCS SP5).

4.1 Cellular kinetic studies

The study was carried out using HeLa cells, cultured in DMEM supplemented with 10% FBS and 1% antibiotic. After 24 h incubation in a 12-well cell culture plate (50000 cells/1800 μL),

the cells were treated with free Dox and Dox conjugates (G-Dox and G-Cys- Fe_3O_4-Dox). The wells were fixed at definite time-points (1, 4, 8 and 24 h) for three consecutive chemo-cycles. Between each chemo-cycle, the old media was replaced with fresh media and cells were incubated for 24 h. Post 24 h, free Dox and Dox conjugates were again added to the wells and fixed at the mentioned time point until three chemo-cycles were complete. Imaging was done under confocal microscope.

4.2 Quantitative nuclear deformation

To study the morphological variations in HeLa cells on repetitive exposure to Dox and its conjugates, three chemo-cycles study was performed to determine the changes in NSA, CSA and CRd of cells. 50,000 cells per well were seeded in 12-well plates and incubated for 24 h at 37 °C. Free Dox, Dox nanocarriers and G-COOH and G-Cys-Fe_3O_4 were added in each well and the cells were fixed at 1, 4, 8 and 24 h for three consecutive chemo-cycles. Between each chemo-cycle, fresh media was added in each well and cells were incubated for 24 h, followed by addition of Dox and its conjugates at their IC_{50} concentrations. Finally, cellular deformation was measured from fluorescence microscope images (>30 cells at each time) of HeLa cells, stained with Eosin and DAPI. NSA, CSA and CRd were calculated using *ImageJ* software.

5. Morphological alterations of HeLa (control) cells, on exposure with G-COOH and G-Cys-Fe_3O_4

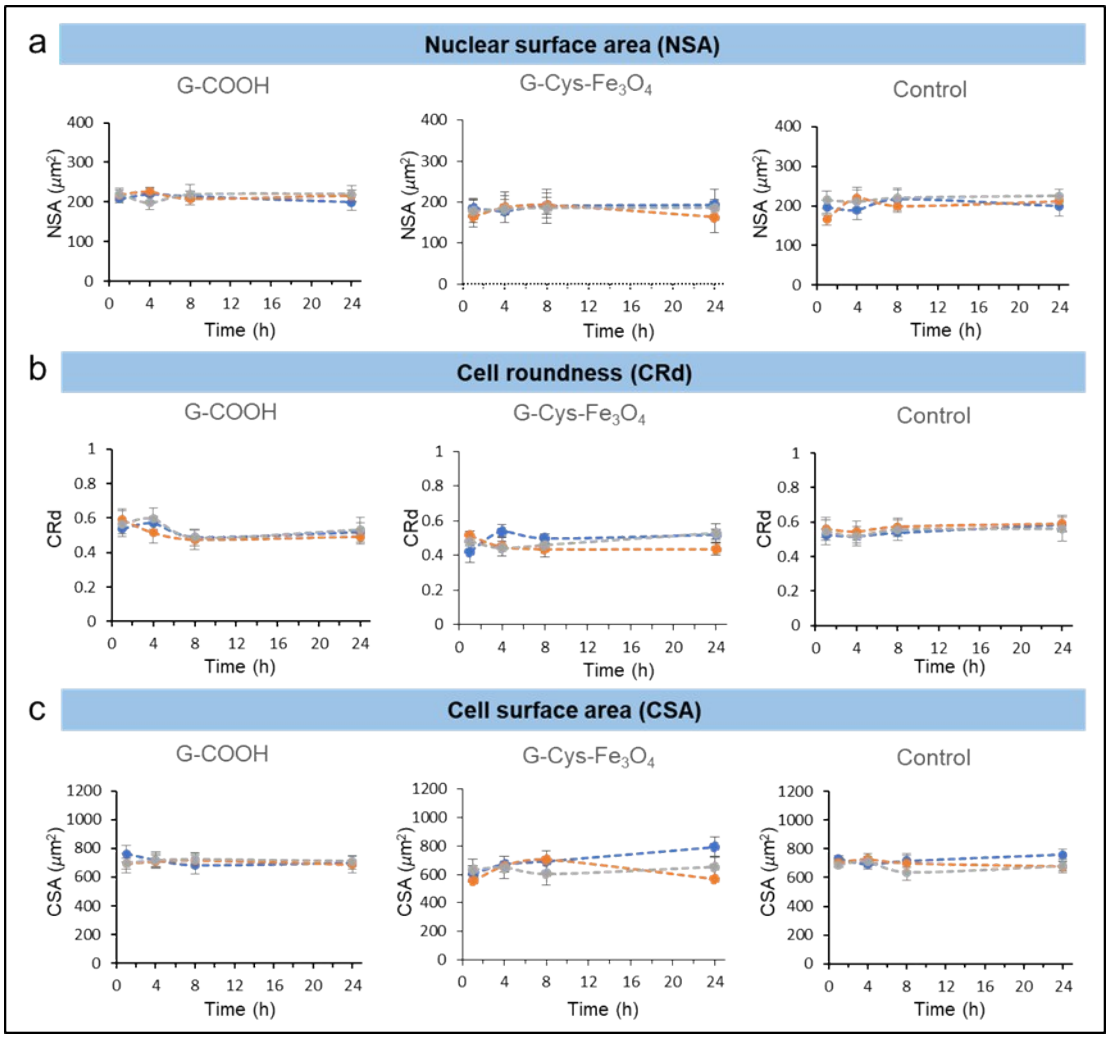

Figure S3. Changes in cell morphological parameters of HeLa cells due to G-COOH, G-Cys-Fe_3O_4 and in control cells. HeLa cells were incubated for 24 h post cell seeding, and thereafter, **(a)** NSA, **(b)** CRd, **(c)** CSA were calculated for three chemo-cycles at 1, 4, 8 and 24 h each.

References

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[2] F. Forohar, C. Whitaker, W. M. Koppes, US 7,807,127 B1, 2010.

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