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# Gd/Hafnium Oxide@Gold@Chitosan Core-Shell Nanoparticles as a Platform for Multimodal Theranostics in Oncology Research

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**Table of content** **Page number**

**Materials**

Hafnium (IV) chloride ($HfCl_4$, 98%), and Gadolinium (III) chloride hexahydrate ($GdCl_3.6H_2O$, 99%), were bought from Sigma Aldrich, India. L-Ascorbic acid ($C_6H_8O_6$, 99.7%), and Sodium sulfide x hydrate ($Na_2S.xH_2O$, 50%) were procured from Merck Chemicals and Reagents, India. Chitosan (50-20 mPa) was obtained from Thermo Fischer Scientific, India. Hydrogen tetrachloroaurate ($HAuCl_4$, 99.9%) was acquired from Alfa Aesar, India. PBS buffer pH 7.4 tablets were procured from Loba Chemie, India. Fluorescein isothiocyanate (FITC, 97%), Mercaptopropanoic acid (MPA, $C_3H_6O_2S$, 99%), and doxorubicin (DOX, 95%) were bought from TCI Chemicals, India. The chemicals were used as procured. All experiments were done in double distilled water.

## Methods

### Drug loading in CAuGH NPs and *In-vitro* drug release

DOX was used as a model drug and was loaded in CAuGH NPs. Briefly, 55 µL aqueous solution of DOX (1 mg/ mL) was dropwise added to a 1.5 mL aqueous dispersion of CAuGH NPs (7.34 mg/ mL) and the sample was kept overnight under constant stirring at 400 rpm. The drug to nanoparticle ratio was held constant at 1:20 (w/w). The obtained DOX loaded CAuGH NPs (DOX-CAuGH NPs) was centrifuged at 10,000 rpm for 15 min. Supernatant (unbound DOX) was collected and the pellet (DOX-CAuGH NPs) was again resuspended in DI water. The process of centrifugation and washing was repeated for three times. The supernatant collected after each step of centrifugation was collectively pooled out and was analysed spectrophotometrically for free DOX by taking absorbance at 482 nm. The absorbance was quantified from the standard curve of aqueous solution of DOX (1–10 µg/mL). DOX-CAuGH NPs were resuspended in DI water and also part of the sample was lyophilized.

Cumulative supernatant pooled out in the above step was analyzed at 482 nm utilizing a UV-Vis spectrophotometer and a standard DOX curve was used to calculate the results. The encapsulation and the loading efficiency of DOX-CAuGH NPs could be determined using the following formula:

$$\%\ Encapsulation\ Efficiency\ (EE) = \frac{Total\ drug - Drug\ in\ supaernatant}{Total\ drug} X\ 100$$

$$\%\ Loading\ Efficiency\ (LE) = \frac{Total\ drug - Drug\ in\ supernatant}{Total\ drug + amount\ of\ CAuGH\ NPs} X\ 100$$

DOX release from DOX-CAuGH NPs was analysed under two different conditions, in 0.1 M PBS (pH 7.4), and in 10 mM GSH, respectively. Briefly, in microcentrifuge tubes, 3 mg of DOX-CAuGH NPs were suspended in 3 mL of PBS (0.1 M, pH 7.4), and 10 mM GSH, respectively. Then, each dispersion was equally filled in three distinct microcentrifuge tubes (for recording the drug release profile in triplicate) and kept on orbital shaker (400 rpm) at 37 ˚C. DOX released from DOX - CAuGH NPs was separated by centrifugation (10,000 rpm for 10 min) at specific time intervals. Further, from each tube the supernatant was collected separately and each tube was again supplemented with respective fresh buffer solution. The DOX released from DOX-CAuGH NPs was analysed spectrophotometrically by taking absorbance at 482 nm and was quantified with the help of DOX standard curve (1–10 µg/mL).

***In-vitro* cytotoxicity analysis and determination of cellular uptake of CAuGH NPs**

The *in vitro* cytotoxicity evaluation of DOX, CAuGH NPs, and DOX-CAuGH NPs was performed against MDA-MB-231 breast cancer cell line with the help of MTT cell viability assay. Briefly, in DMEM media supplemented with 10% FBS, MDA-MB-231 cells were seeded in a 96-well plate at a cell density of 1 x $10^4$ cells/well and incubated at 37 ˚C and 5% $CO_2$. After an incubation period of 24 h, the media from each well of the 96- well plate was separated and exchanged with fresh media. Treatment in triplicate was then given to cells using varied concentrations of DOX, CAuGH NPs, and DOX-CAuGH NPs. The treatment was followed by incubating the cells for another 24 h. Non-treated cells were used as a control. After 24 h, 10 µL MTT solution (5 mg/mL) made in 0.1 M PBS was added to each well followed by another 2 h of incubation. Insoluble formazan crystals were seen in each well which were then dissolved with DMSO (100 µL) and the absorbance was checked at 570 nm. The following equation was used to determine the percent cell viability:

$$\%\ cell\ viability = \frac{absorbance\ of\ treated\ cells}{absorbance\ of\ control\ cells} * 100$$

The cellular uptake of CAuGH NPs was examined using MDA-MB-231 cell line. Briefly, MDA-MB-231 breast cancer cells were cultured on a coverslip placed in 6 well plate at a density of 1 x $10^6$ cells/well in DMEM media complemented with 10% FBS. Cells were incubated for 24 h in an incubator at 37 ˚C and 5% $CO_2$. After an incubation period of 24 h, media from each well was removed and replenished with fresh media. Each cover slip was incubated with FITC-tagged CAuGH NPs (1.4 µg/mL) for 4 h with a total volume of 100 µL/well. Following incubation, cells were washed thrice with an ice cold 0.1M PBS and fixed for 10 min at 4 ˚C using 4% paraformaldehyde followed by washing thrice using ice cold solution of 0.1 M PBS. Finally, cells were affixed with mounting material to a glass slide accompanied with DAPI (100 ng/mL). The cellular distribution and uptake of CAuGH NPs was investigated in FITC and DAPI channels.

**ROS estimation analysis**

For estimating ROS generation upon gamma irradiation, 1 x $10^4$ cells/well (MDA-MB-231 breast cancer cells) in DMEM media and 10% FBS were seeded in a 96 well plate followed by their incubation at 37 ˚C and 5% $CO_2$ for 24 h. *In vitro* irradiation of MDA-MB-231 cells treated with CAuGH NPs was accomplished with the aid of gamma radiation provided by Cobalt-60 irradiation chamber running at 6 mV with a dosage rate of 0.812 Gy/min. Non-treated MDA-MB-231 cells were kept as control. A distance of 80 cm was maintained between the gamma source and the cell plates. Before and after irradiation, the intracellular ROS levels generated in MDA-MB-231 cells were recorded using a colorimetric assay based on 2',7'-dichlorodihydrofluorescein diacetate (DCFHA-DA), a fluorescent dye. In brief, post treatment of 24 h, the old media was rejected and the cells were supplied with fresh media. Next, 2 µL of DCDHF-DA (0.2 µg/µL) dye was supplemented to each well 45 min prior to irradiation (0.5 Gy and 5 Gy). Each well's fluorescence emission spectra were obtained at 535 nm after the dye was excited at 485 nm at predetermined intervals. The plates of MDA-MB-231 cells without irradiation and CAuGH NPs treated MDA-MB-231 cells were kept as control and ROS generation was simultaneously assessed for them at specified time intervals.

***In vitro* evaluation of CAuGH NPs as contrast agent for magnetic resonance imaging (MRI)**

The samples for *in vitro* MRI were prepared in phantom gel (1.5 mL of 2% low melting agarose solution) with the iron concentration ranging from of 0-40 µg/ mL. In Bruker 7 T MRI scanner, MRI studies were carried out at 25 °C. In the MRI scanner, spin-echo sequences with echo times ranging from 8 to 72 milliseconds (ms) and repetition times of 400 to 5500 ms were run on microcentrifuge tubes having formulations. Using ImageJ software, the data were examined. At different echo times, signal intensities were assessed from regions of interest (ROI) in each microcentrifuge tube over the image set. The decay curves were created by plotting the results as a function of echo times. Using Origin 2020b (ORIGIN Lab), the curves were fitted utilizing the monoexponential decay curve equation. Following equation was used to get the values of $T_1$ relaxation times:

$R_1 = A + C [\exp (-R_1 * T_R)]$

From the slope of the plot between $R_1$ (or $1/T_1$) and the concentration of gadolinium, $T_1$ relaxivity ($r_1$) was estimated, taking the protocol reported elsewhere [1].

***Instrumentation:***

Zetasizer Nano ZS (Malvern Instruments Ltd.) was used to record the hydrodynamic size distribution ($D_H$) and polydispersity index (PDI) of AuGH NPs and CAuGH NPs.

UV-Vis absorption spectra were recorded on Multimode microplate reader (Tecan infinite M200 Pro) between 400-800 nm range.

Qualitative powder X-Ray diffractometer (XRD) measurements were done using Smart Lab 9kW rotating anode X-ray diffractometer (Rigaku Corporation) over a 2θ range of 10˚- 80˚ using Cu Kα irradiation source of 0.1542 nm.

FTIR analysis was done using L1600312 spectrum TWOLITA/ZnSe (Agilent Technologies).

Thermogravimetric analysis was done on STA 449 F1 jupiter (NETZSCH Geratebau GmbH) in a temperature range of 30 ˚C to 900 ˚C with a heating rate of 20 ˚C/min under $N_2$ atmosphere.

X-Ray Photoelectron analysis was done on XPS Thermofisher Scientific NEXSA photoelectron spectrometer using Al Kα (1486.6 eV). Data analysis was done utilizing Avantage software.

Field emission scanning electron microscopy (FESEM) measurements were carried out using Nova Nano SEM-450 FESEM instrument. Elemental analysis (EDAX) of CAuGH NPs was done using JEOL JSM-7600F FESEM.

Atomic force microscopy (AFM) was done using Dimension ICON PT, Bruker instrument.

High resolution transmission electron microscopy (HRTEM) was performed using Technai G2s- Twin, FEI instrument functioning at 200 kV. Sample preparation was done by drop casting an aqueous dispersion of CAuGH NPs on copper grid coated with carbon and dried at room temperature. ImageJ software was used for calculating the average size of CAuGH NPs and histogram was plotted using Origin 2018 64bit software.

Confocal laser scanning microscopy (CLSM) (Leica, SP8) was carried out for cellular uptake studies.

MRI was done with the help of 7 T Bruker Biospec MRI scanner (Bruker BioSpin MRI GmbH, Germany) having a uniform external magnetic field. Different concentrations of CAuGH NPs in an aqueous solution of low melting agar (2%) was prepared and analyzed in MRI. The data obtained was analysed using internal software of Biospec MRI. With eight echo times (TE) ranging from 8 to 120 milliseconds (ms) and a repetition time ($T_R$) of 5500 ms, the $T_1$ relaxation time was measured.

***Statistical analysis:***

Statistical analysis was performed utilising the software Graph Pad Prism (version 5.1). The data were statistically analyzed using one-way analysis of variance (ANOVA) at $p < 0.05$, 0.01, and < 0.001 level of significance.

## Results

### UV-Vis spectra

UV absorbance scan was done to validate the development of gold shell over GH NPs by making use of SPR effect of gold. **Figure S1** displays a comparison of UV-Vis spectra of bare $HfO_2$ NPs, GH NPs, AuGH NPs, and Au NPs. In case of bare $HfO_2$ NPs, and GH NPs no peak was found in the experimental wavelength range. In contrast, AuGH NPs exhibited a red shifted SPR peak at about 539 nm as compared to Au NPs ($\lambda_{max}$ 529 nm), thus confirming the formation of thin gold shell over GH NPs.

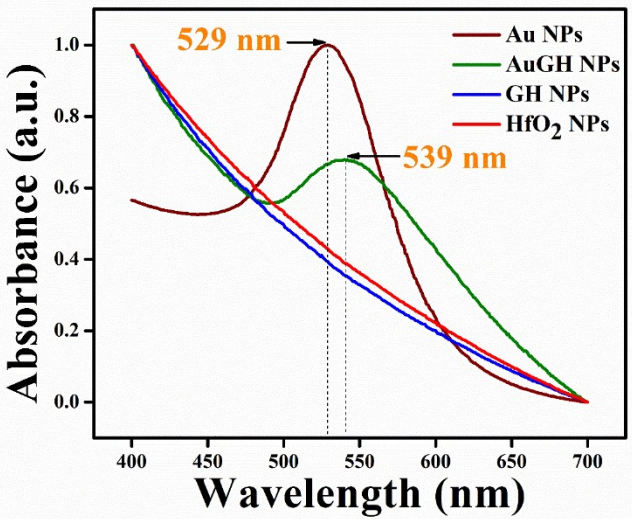


**Figure S1**. UV-Vis spectra of gold nanoparticles (Au NPs) (brown), AuGH NPs (green), GH NPs (blue), and bare $HfO_2$ NPs (red).

**DLS and Zeta studies**

The hydrodynamic diameter and zeta potential of the CAuGH NPs was analyzed at different pH conditions. It was found out that in acidic pH, the hydrodynamic size was 1162 nm and 1248 nm and zeta potential was 3.7 and 2.6 at pH 2 and 4 respectively, which could be attributed to the protonation of all the amine groups present on the chitosan. As a result, repulsive forces are encountered by CAuGH NPs due to which, a higher hydrodynamic diameter is observed. Further increasing the pH close to the pKa value of chitosan which is around 6.5, a decreasing trend in the hydrodynamic diameter was observed. This could be ascribed to the near similar ration of protonated and deprotonated amines. Further increase in the pH from 6 to 10 results in an increase in the size of CAuGH NPs which could be due to the complete deprotonation of all the amine groups present in the system. This is also reflected by the negative value of zeta potential which is -9.7 and -7.5 at pH 8 and pH10 respectively.

The stability of the CAuGH NPs was also analyzed over time at physiological pH by measuring the hydrodynamic diameter and zeta potential. It can be seen from the graph that the particle size is showing variability in particle size as well as zeta potential, which suggests that the particles are undergoing some dynamic adjustments resulting in higher hydrodynamic diameter on day 3 which further reduces on day 5.

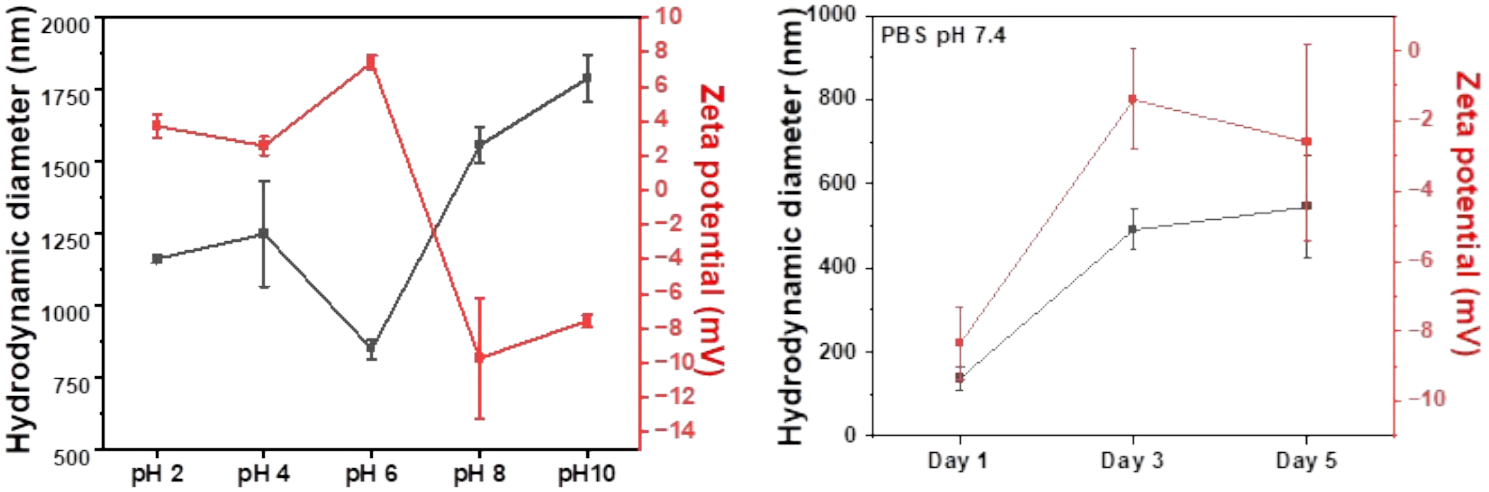


**Figure S2**. Variation in hydrodynamic size and zeta potential values with pH and time (at pH 7.4).

**Stability of the CAuGH NPs in 5 % BSA**

The stability of CAuGH NPs in 5 % BSA was checked with the help of UV-Vis spectroscopy. In the graphical representation, a shoulder appeared at around 550 nm with negligible time dependent changes in the peak position over the passage of time. It is speculated that minimal protein corona formation occurs on the surface of the designed NPs which could be due to the minimal electrostatic interaction between BSA and CAuGH NPs. Further, it is hypothesized that due to the thiolation of chitosan, the overall positive charge associated with chitosan is reduced which restricts its electrostatic interaction with negatively charged BSA, resulting in minimal corona formation thereby negligible aggregation which reflects the stability of CAuGH NPs.

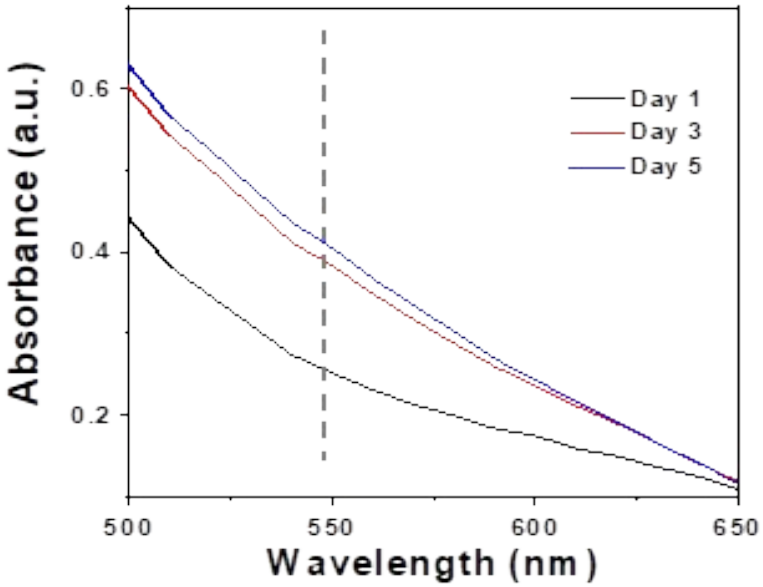


**Figure S3**. UV-Vis of CAuGH NPs in 5 % BSA.

**EDAX of CAuGH NPs**

EDAX measurement showed the presence of Gd, Hf, and Au in the sample confirming the successful doping of Gd in hafnium oxide nanoparticles.

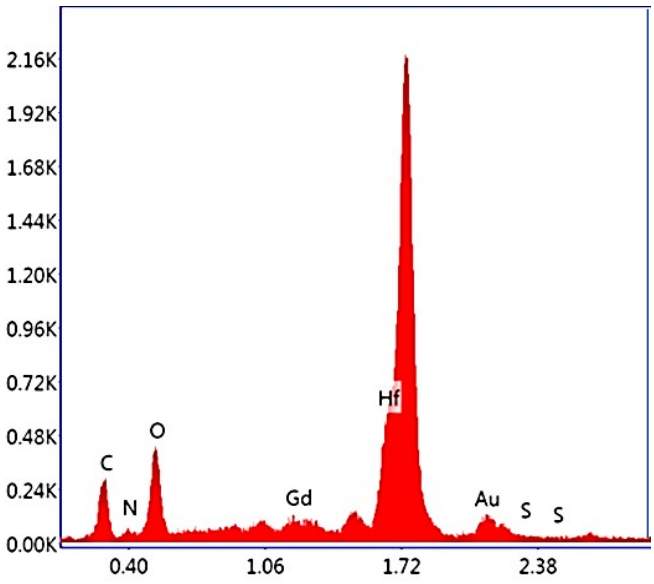


**Figure S4.** EDAX displaying elemental composition of CAuGH NPs.

**XPS analysis**

XPS was done to confirm the chemical composition of CAuGH NPs **(Figure S2A)**. The peaks at 1187.7 eV, and 1219.9 eV correlated to the 3d spin-orbit doublet peaks of Gd $3d_{5/2}$ and Gd $3d_{3/2}$ respectively **(Figure S2B)**. Further, O 1s peak was deconvoluted into two spin-orbit doublet peaks of 532 eV and 530 eV which referred to C=O and Hf-O present in ascorbic acid and $HfO_2$ respectively **(Figure S2C)** [2, 3]. Moreover, C 1s peak upon deconvolution presented two doublet peaks of O-C=O (ascorbic acid) and C-C (thiolated chitosan used for stabilization) with binding energy 288.8 eV and 285.7 eV respectively **(Figure S2D)**. Also, Gd 4d peak was due to the doping in $HfO_2$. Two spin-orbit doublet peaks are formed from the deconvoluted Gd 4d peak i.e. $4d_{3/2}$ and $4d_{5/2}$ with binding energy of 148.9 eV and 143.3 eV respectively which are characteristic peaks of Gd **(Figure S2E)** [4]. **Figure S2F** displays the typical spin-orbit doublet peaks of Au 4f. Here, the peaks at 87.86 eV and 84.23 eV corresponded to Au $4f_{5/2}$ and Au $4f_{7/2}$ respectively which was ascribed to $Au^0$ ground state of gold shell [2]. The peak of Hf was attributed to hafnium oxide core which upon deconvolution resulted in two spin-orbit doublets for Hf $4f_{5/2}$ (18.51 eV) and Hf $4f_{7/2}$ (17.01 eV) corresponding to fully oxidized hafnium ($Hf^{4+}$) and hafnium suboxides ($Hf^{x+}$) respectively **(Figure S2G)** [3]

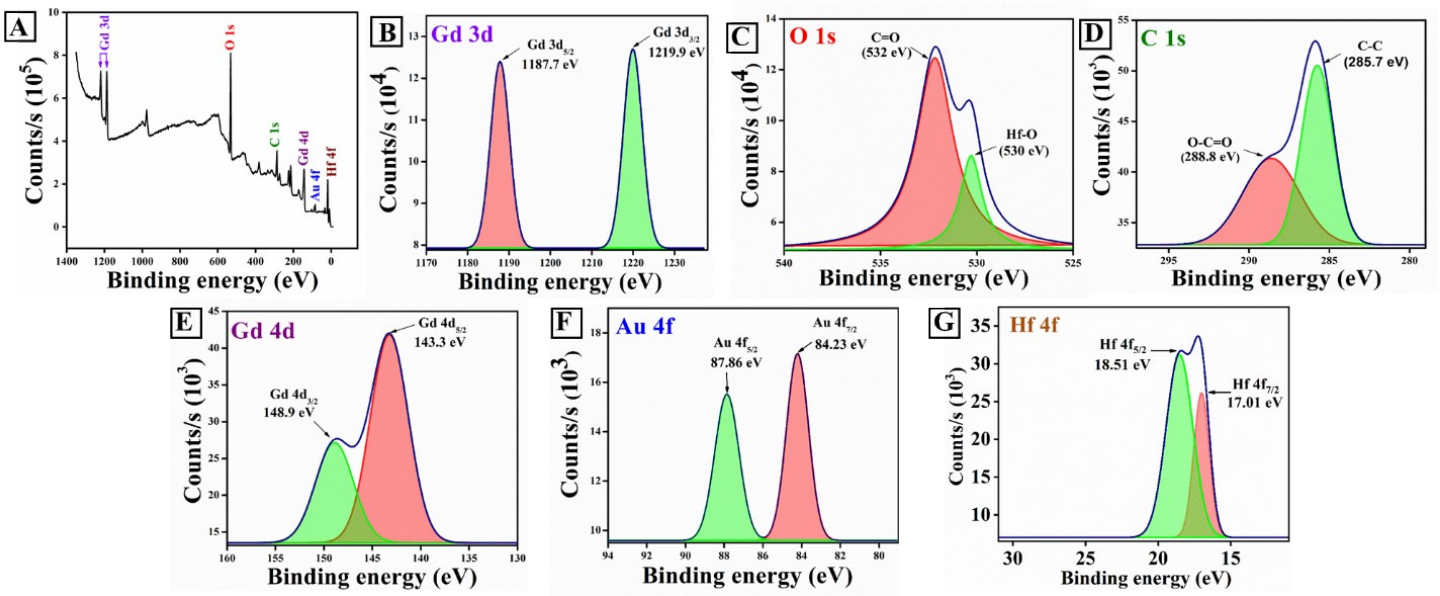


**Figure S5.** XPS spectra of CAuGH NPs showing the binding energy values of (A) all the elements combined, (B) gadolinium 3d, (C) oxygen 1s, (D) carbon 1s, (E) gadolinium 4d, (F) gold 4f, and (G) hafnium 4f.

**Cell viability studies**

The cell viability of CAuGH NPs and DOX-CAuGH NPs was checked towards HEK293T cell line. It was observed that upto a dose rate of 5 µg/mL, almost 90 % and 80 % of cells are viable in case of CAuGH NPs and DOX-CAuGH NPs respectively.

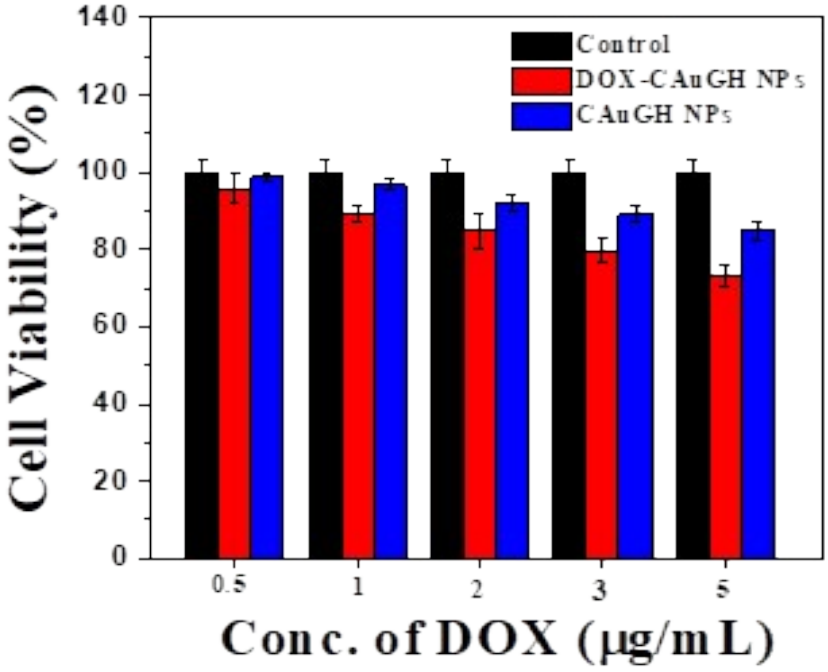


**Figure S6.** Cell viability of CAuGH NPs (blue) and DOX-CAuGH NPs (red) towards HEK293T cell line.

**Cellular uptake studies**

The efficiency of CAuGH NPs for cellular internalization was evaluated using CLSM on MDA-MB-231 breast cancer cells. The cellular uptake was recoded at 15 min and 4 h post treatment of MDA-MB-231 cells with FITC tagged CAuGH NPs **(Figure S4)**. For the first 15 min, the uptake of CAuGH NPs by MDA-MB-231 cells was lesser which is clearly evident with less fluorescence signal in the green channel. However, distinctly detectable signal in the green channel could be seen after 4 h treatment. This increment in cellular internalization with the passage of time confirms the time dependent cellular uptake of CAuGH NPs.

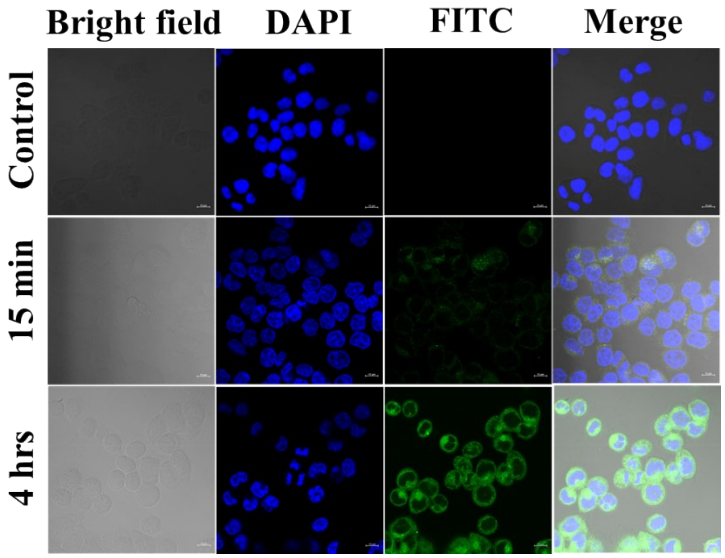


**Figure S7**. Cellular uptake of FITC tagged CAuGH NPs in MDA-MB-231 cells in DAPI and FITC at 15 min and 4 h.

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