Supplementary Information

Engineering the Biotin Anchored-MWCNT as a Superb Carrier for Facile Delivery of Potent Ru(II)-N^N Scaffold in Breast Cancer Cells‡

Lavanya Thilak Babu,[¥] Nilmadhab Roy,[¥] Sreejani Ghosh Tiasha Dasgupta, Tamizhselvi Ramasamy and Priyankar Paira*†

1. CHARACTERISATION

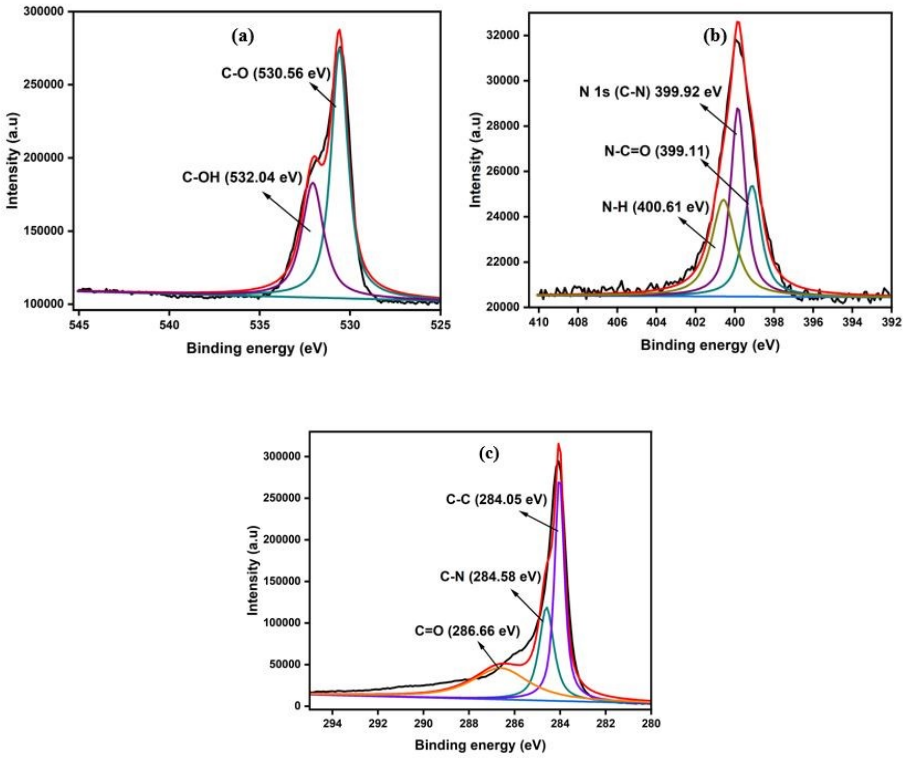

Fig. S1 XPS spectra of (a) O 1s MWCNT-COOH, (b) N 1s MWCNT-TEG-NH_2 and (c) C 1s MWCNT-TEG-Biotin

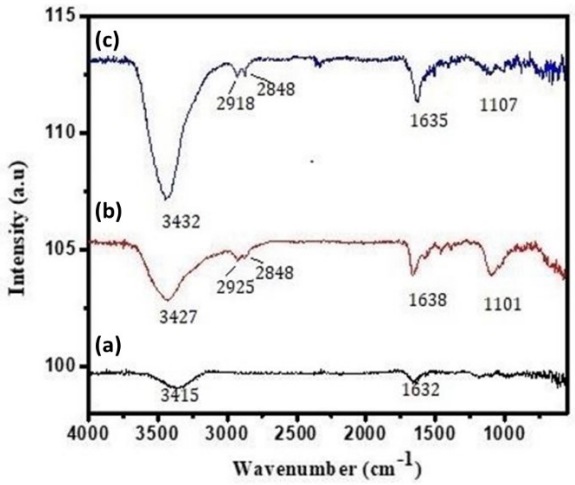

Fig. S2 FTIR spectra of (a) MWCNT-COOH, (b) MWCNT-TEG-NH_2, and (c) MWCNT-TEG-Biotin

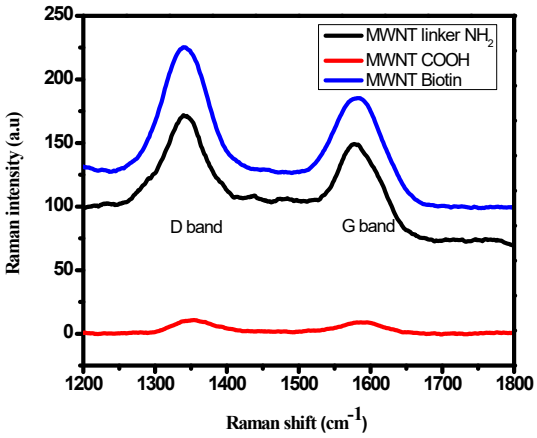

Fig. S3 Raman spectra of MWCNT-COOH, MWCNT-TEG-NH_2, and MWCNT-TEG-Biotin

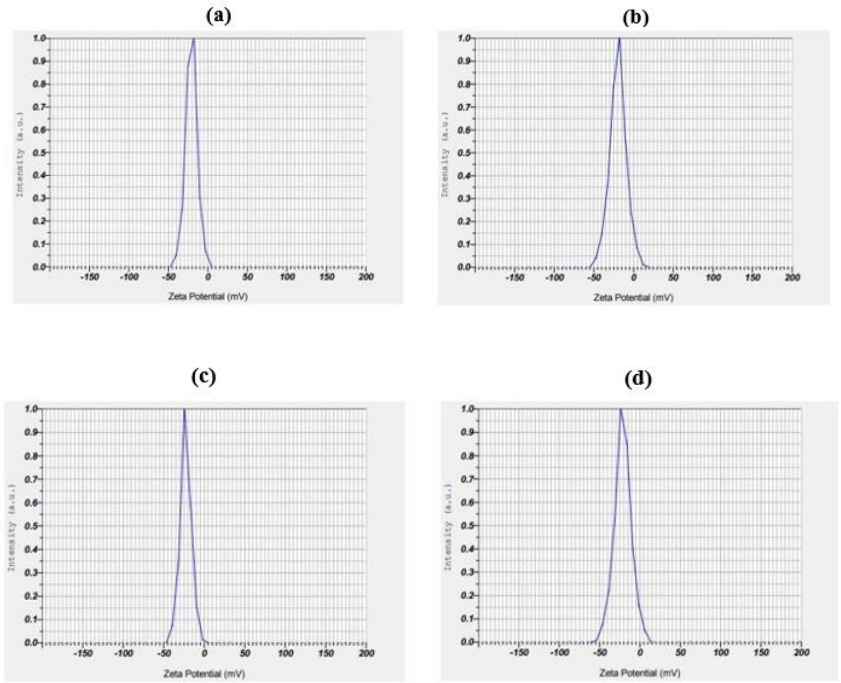

Fig. S4 Zeta potential analysis of MWCNT-COOH, MWCNT-TEG-NH_2, MWCNT-TEG Biotin, and complex **7a** loaded in MWCNT-TEG-Biotin

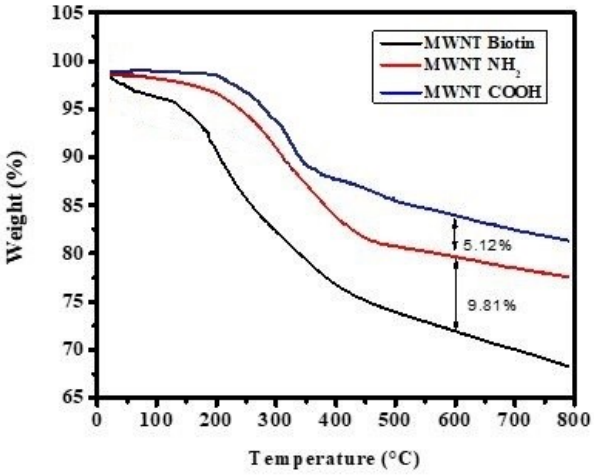

Fig. S5 TGA spectra of MWCNT-COOH, MWCNT-TEG-NH_2, and MWCNT-TEG-Biotin

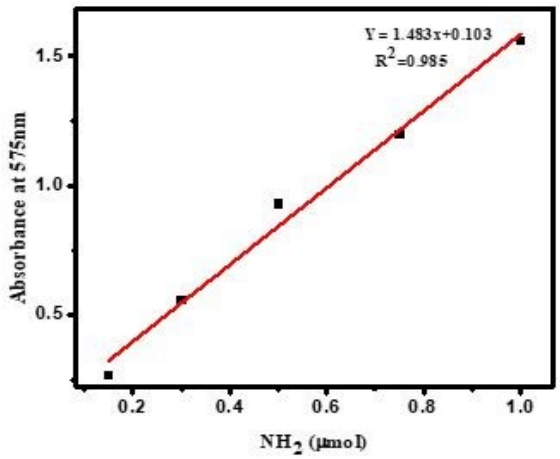

Fig. S6 Standard graph of using the Kaiser test.

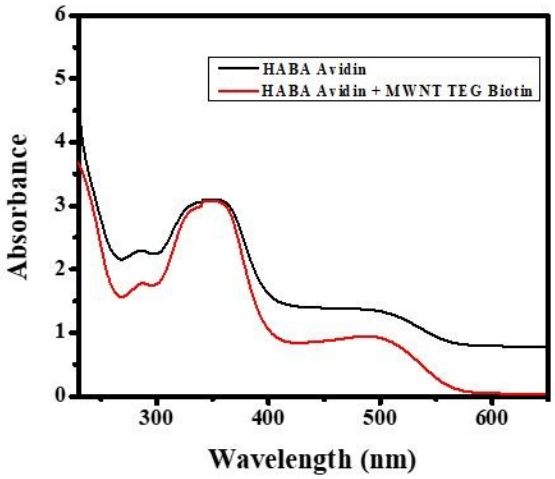

Fig. S7 UV Visible spectrum of HABA Avidin and HABA Avidin along with MWCNT-TEG-Biotin

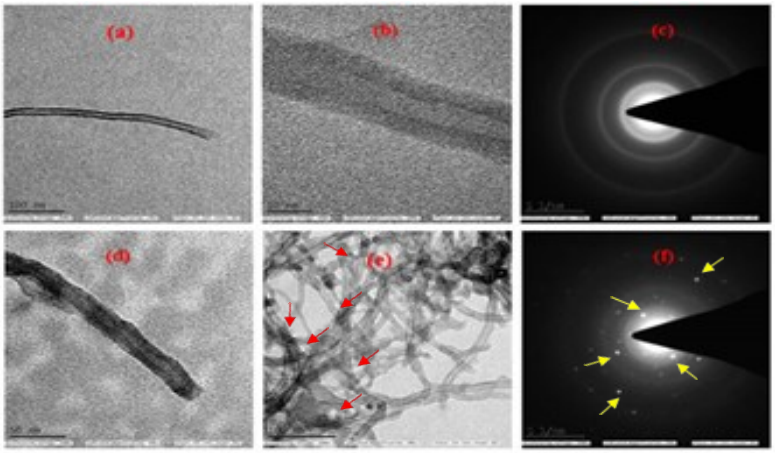

Fig. S8 (a), (b) TEM images of MWCNT-COOH and (d), (e) complex **7a** loaded in MWCNT-TEG-Biotin. (c) SAED images of MWCNT-COOH and (f) complex **7a** loaded in MWCNT-TEG-Biotin.

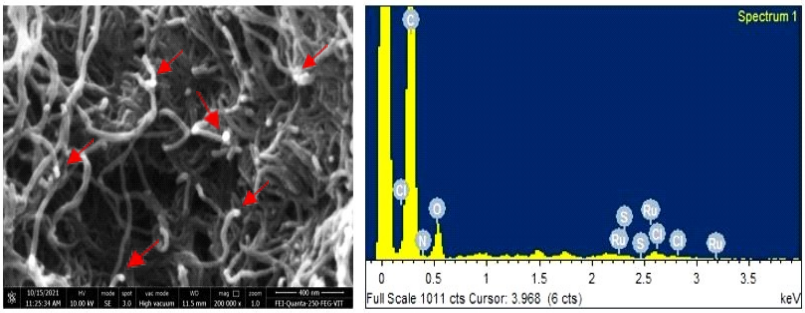

Fig. S9 (a) SEM image of complex **7a** loaded in MWCNT-TEG-Biotin and (b) EDX image of complex **7a** loaded in MWCNT-TEG-Biotin

2) BIOLOGICAL STUDIES

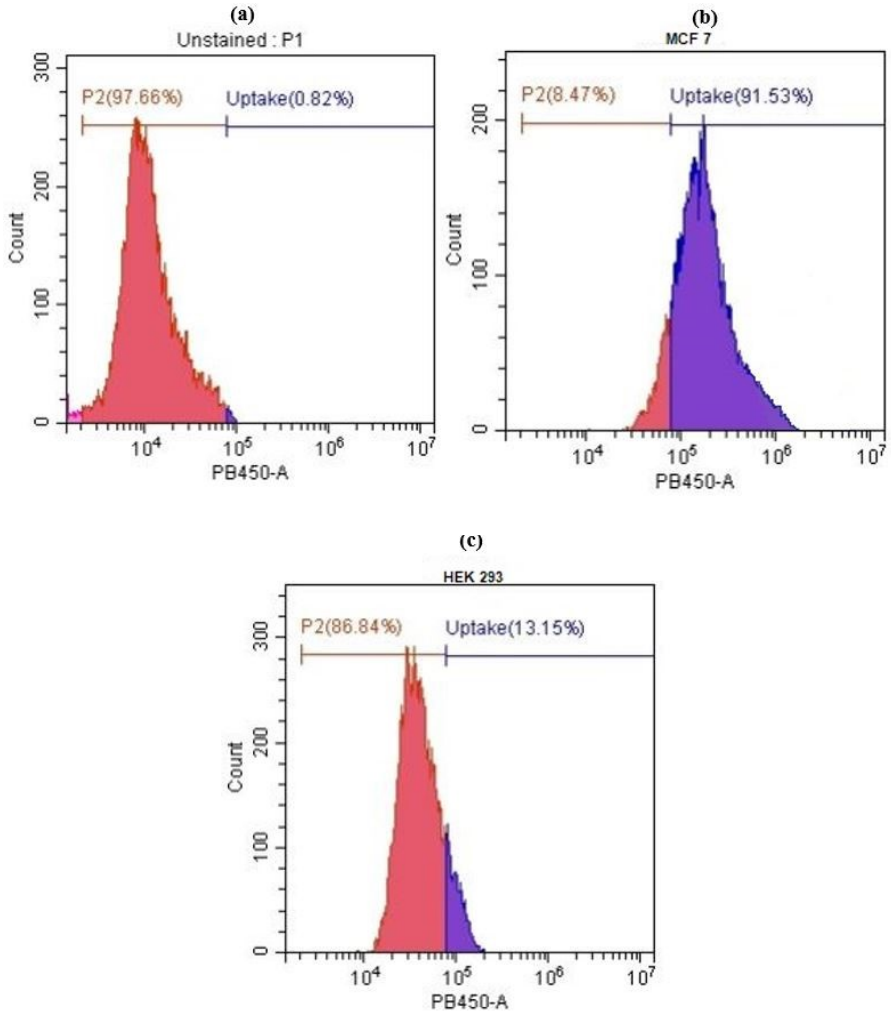

Fig. S10 Quantification of Cellular Uptake of a) control and b) complex **7a** loaded in MWCNT-TEG-Biotin in MCF 7 cells and c) complex **7a** loaded in MCWNT-TEG-Biotin in HEK 293 cells.

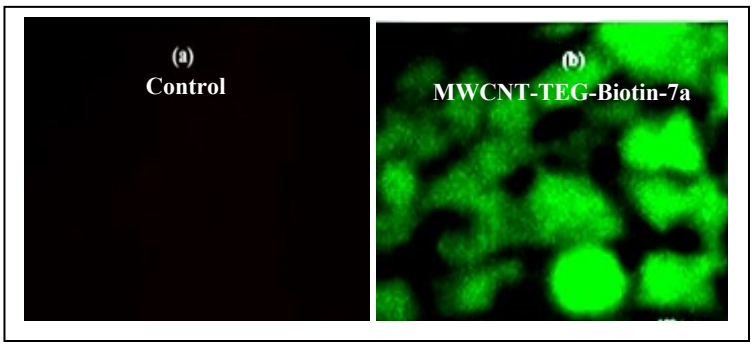

Fig. S11 Fluorescence images of a) control and b) complex **7a** loaded in MWCNT-TEG-Biotin in MCF-7 cells.

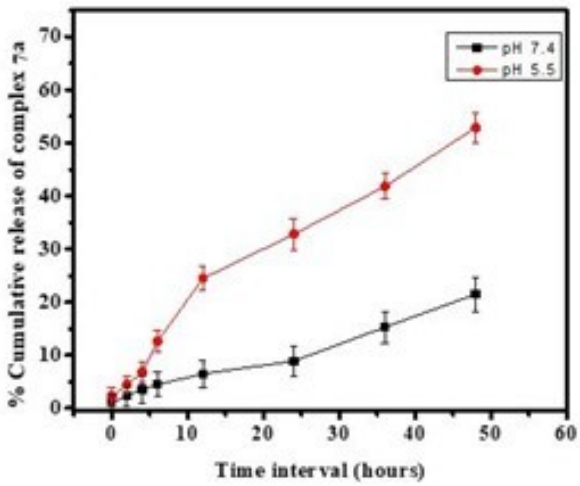

Fig S12 *Invitro* complex **7a** release

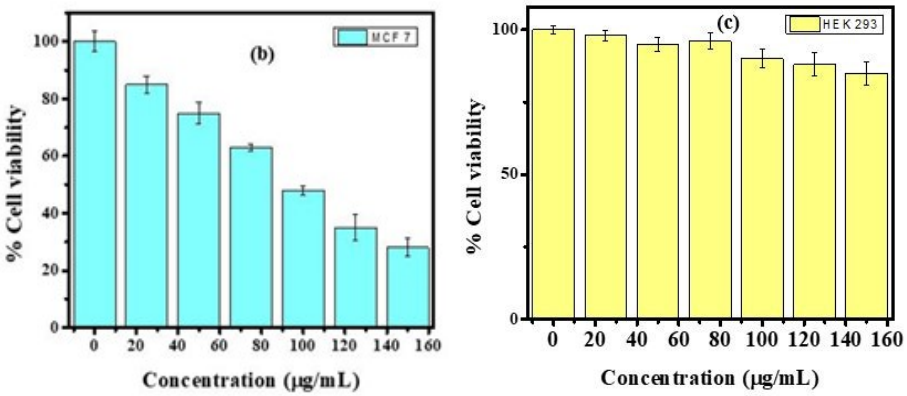

Fig. S13 Cytotoxicity profile in MCF 7 and HEK 293 cell

Table S1 Cell cycle analysis in MCF 7

FACS analysis of Cell cycle arrest in MCF-7 cells				
Samples	**SUBG$_0$**	**G$_0$/G$_1$**	**S**	**G2/M**
Control	2.66	67.42	18.95	8.81
25µg/mL	2.44	93.27	4.39	1.19

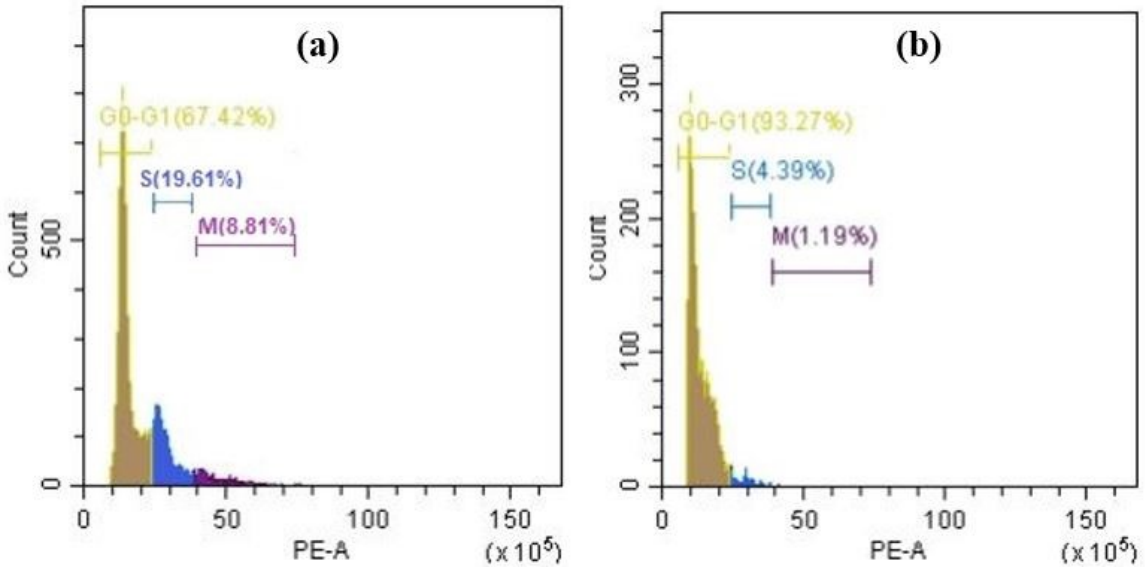

Fig. S14 Cell cycle analysis in MCF 7 a) control b) treated with 25µg of MWCNT-TEG-Biotin loaded with complex **7a**

Experimental Section

Materials and methods

In this study, high-quality reagents and solvents of commercial grade were employed. All chemical and biochemical substances were sourced from Sigma-Aldrich Chemical Ltd, Merck. Cell lines utilized in the experiments were obtained from NCCS, Pune. The DMEM medium, along with 1% penicillin, streptomycin, and 1% Glutamax, were procured from Gibco. Fetal bovine serum at 10% concentration and 0.25% trypsin-EDTA were acquired from Himedia and Thermo Fisher Scientific, respectively. X-ray photoelectron spectroscopy (XPS) analysis was carried out using the Krotas analytical instrument, specifically the ESCA 3400 model, equipped with Dual Mg/Al anodes. The instrument operated at a voltage of 12 kV with a current of 25 mA, all conducted under ultra-high vacuum conditions. Functional group identification was performed using the FT-IR instrument Perkin Elmer-RX1. The Raman spectroscopy was conducted using the BRUKER RFS 27 MultiRAM FT Raman Spectrometer. The Zeta potentials of all the samples were measured using Litesizer 500 Zeta Potential Analyzer. Q500 Hi-Res Thermogravimetric analyzer was utilized for the TGA study. All the UV-visible spectra were recorded on a JASCO V-760 spectrometer. For scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) analysis, a Hitachi S-3400N SEM machine was employed. The morphology of the nanoparticles, as well as transmission electron microscopy (TEM) and selected area electron diffraction (SAED) images, were examined using the high-resolution transmission electron microscope JEOL 3010. An Elisa reader and 96-wellplates were utilised for the MTT assay.

Functionalising MWCNT-COOH using amine-terminated TEG groups

To introduce amine-terminated triethylene glycol (TEG) groups, multi-walled carbon nanotubes functionalized with carboxylic acid groups (20 mg of MWNT-COOH, with a diameter of 9.5 nm and a length of 1.5 μm) were subjected to a reaction. This modification involved treating the surface of MWNT-COOH with 2,2'-(ethylene dioxy) bis(ethylamine) (5 mL, 8 mmol) in the presence of HATU (45.6 mg, 120 mmol) as a coupling agent and DIPEA (24 μL, 180 μmol) at 60°C for 24 hours. Upon completion, the reaction mixture was extracted with dimethylformamide (DMF) (3 mL) to eliminate unreacted organic compounds. The remaining mixture containing multi-walled carbon nanotubes (MWCNTs) was thoroughly washed with DMF, methanol, dichloromethane (DCM), and finally, deionized water. Subsequently, MWCNT-TEG-NH_2 was retrieved and air-dried for further use.

Preparation of Biotin-functionalised MWCNT

The functionalization process of MWCNT-TEG-NH_2 with biotin involved combining biotin (8 mg, 22 mmol) with MWCNT-TEG-NH_2 (20 mg, 168 μmol of free NH_2 equivalents), along with EDC (2.7 mg, 22 mmol) and NHS (8.5 mg, 44 mmol). The reaction took place in the presence of a catalytic amount of DMAP and DIPEA in dimethylformamide (DMF) (2 mL) for 24 hours at room temperature, resulting in the formation of MWCNT Biotin. Subsequently, the residual mixture containing MWCNTs underwent thorough washing with DMF, methanol, dichloromethane (DCM), and finally, deionized water. Ultimately, MWCNT-TEG-Biotin was retrieved and air-dried for subsequent applications.

Loading of potent drug complex 7a 2-(2-(4-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)butoxy)phenyl)benzo[d]thiazole- Ruthenium (II) arene in MWCNT -TEG-Biotin.

A dispersion of 10 mg of MWNT-TEG-Biotin in 5 mL of distilled water was prepared and subjected to bath sonication for a brief period before utilization. Simultaneously, a solution of 2-(2-(4-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)butoxy)phenyl)benzo[d]thiazole-Ruthenium (II) arene complex (10 mg) was dissolved in 0.5 mL of methanol. The thorough mixing of MWNT-TEG-Biotin and complex 7a was achieved through sonication and continuous stirring over 48 hours. The separation of unbound complexes was accomplished by centrifuging the MWNT-TEG-Biotin mixture, followed by careful washing of the pellet with deionized water to eliminate loosely bound complexes.

The amount of drug complex 7a loading can be determined from the following equation

$$\frac{(O.D\ of\ the\ initial\ amount\ of\ complex\ 7a) - (O.D\ of\ the\ unbound\ complex\ 7a)\ X\ 100}{(O.D\ of\ the\ initial\ amount\ of\ complex\ 7a)} \quad ...(1)$$

Kaiser test

To quantitatively assess the quantity of NH_2-TEG-NH_2 linker conjugated to MWCNT-COOH, the Kaiser test was employed following a standard protocol. This test utilised solutions compromising phenol dissolved in ethanol, ninhydrin in ethanol, and KCN in pyridine. A volume of 500µL from each solution was introduced to 1 mg of MWCNT-TEG-NH_2 to detect free primary amines. The concentration of primary amines in the sample was determined by measuring absorbance at a wavelength of 505nm, using standard plots of Ethylenedioxy bis(ethylamine) as reference, in a UV-visible spectrophotometer.

Biotin-concentration Quantification using Biotin-Assay

The quantification of the biotin content linked to MWNT-TEG-Biotin was conducted through the utilization of HABA/Avidin reagent and UV-Vis spectrophotometry. Approximately 1 mg of MWNT-TEG-Biotin was dispersed in 1 mL of deionized water. A stock solution of HABA/Avidin was prepared by dissolving 20 mg in 1 mL of deionized water. For biotin detection in the sample, approximately 100 µL was withdrawn from the stock, and 900 µL of the reagent solution was added. The absorbance was subsequently recorded at a wavelength of 500 nm.

The assessment of biotin concentration was calculated using the given equation:

$$\Delta A500 = 0.9 \times (HABA/Avidin\ absorbance) - (HABA/Avidin + sample) \quad ...(2)$$

$$Biotin\ concentration\ (\mu M) = \frac{(\Delta\ A500)}{34} \times 1000 \quad ...\ (3)$$

***Invitro* study to quantify complex 7a release**

The in-vitro release of complex **7a** from MWCNT-TEG-Biotin was achieved through the dialysis bag diffusion method. Prior to utilization, the dialysis bag underwent an overnight soaking in double-distilled water. The release medium, phosphate-buffered saline (PBS) at pH 7.4 and 5.5, was contained in a beaker. A dialysis bag with a molecular weight cut off of 14 kDa was filled with approximately 5 mg of MWCNT-TEG-Biotin loaded with complex **7a**, dissolved in 10 mL of distilled water. Subsequently, the dialysis bag was immersed in the release medium and subjected to agitation at 100 rpm. At specified time intervals, 1 mL of the solution from the release medium was withdrawn, and simultaneously, an equivalent volume of buffer was introduced to the beaker. The absorbance was then measured at 328 nm using a UV/Vis spectrophotometer.

Detection of Mitochondrial Membrane Potential:

MCF-7 were seeded in 6 wells plate at a density of 1×10^3 per well. After cells reached 70% confluency, cells were treated with complex and carrier alone for 24 hours. For pH dependent study media pH was monitored and adjusted with 1N HCl to 5.7. After 24 hours the media was

removed and washed with ice cold 1X PBS. After that cells were treated with Rhodamine B at a concentration of 20 nM for 10 minutes at dark. After that cells were washed with PBS three times and captured in confocal microscope (Olympus FluoView FV3000).

Scheme: S1 Schematic depiction of Synthesis of (a)MWCNT-TEG-NH_2 (b) Biotine conjugated MWCNT and (c) loading of potent drug complex in the Biotine functionalised MWCNT.