Electronic Supplementary Information

Moiré Superlattices of Copper Nanocluster Assemblies for Cancer Theranostics

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Contents

Experimental Section:

Materials. Copper (II) nitrate trihydrate (SigmaAldrich), 3-mecaptopropanoic acid (MPA, Sigma-Aldrich), 4- mercaptobenzoic acid (MBA, Sigma-Aldrich), zinc acetate dihydrate (Merck), N, N dimethylformamide (DMF, Merck), triphenylphosphine (TPP, Sigma-Aldrich),Thiazolyl blue formazan (MTT,HiMedia), 2′,7′-Dichlorofluorescin diacetate (DCF-DA, Sigma-Aldrich), and Hydrogen Peroxide (H_2O_2, Merck) were purchased and used without further purification.Elixgrade water from a Milli-Q purification system was used for the experiments.

Synthesis of CuNCs. Copper nanoclusters (CuNCs) were synthesized by adding 2.4 mL of 10 mM copper nitrate solution and 1.6 mL of 0.11 M MPA solution to a 20 mL round bottom flask containing DMF as a solvent at 0 °C. The mixture was stirred for 5 min, followed by the addition of 6 mg of MBA. The solution was stirred for another 30 min, resulting in a pale yellow CuNC dispersion. The dispersion was stored at 4 °C for further experimentation.

Synthesis of hexagonal Zn-CuNCs. 1.5 mL of a prepared CuNC dispersion was added to 1.5 mL of water. After that, 350 µL of a 100 mM zinc acetate solution was added, leading to the formation of a white dispersion. The dispersion was then centrifuged at 10,000 rpm for 10 min, resulting in a white pellet. The pellet was collected and dispersed in water for further experiments.

Synthesis of TPP added moiré Zn-CuNCs. A solution of 5 mM TPP was prepared in acetone to be used as a stock solution. In a vial, 2 mL dispersion of the Zn-CuNCs (with a concentration of Cu metal of about 0.5 mM) was taken and reacted with 0.83 mM concentrations of TPP by adding 400 µL of the TPP solution to the vial. The reactant mixture was kept at room temperature for 5 min before being centrifuged at 15,000 rpm for 15 min. The centrifuged products were collected for further experiments.

Optical measurements. Photoluminescence spectra for all the samples were recorded using the Agilent Cary 100 UV−visible (UV-Vis) spectrophotometer and the HORIBA FluoroMax-4 spectrofluorometer, respectively.

Transmission electron microscopic (TEM) analysis and selected area electron diffraction (SAED) analysis. TEM and SAED of CuNCs, hexagonal Zn- CuNCs and moiré Zn-CuNCs

were performed in JEOL JEM 2100 and JEOL JEM 2100F at a maximum accelerating voltage of 200 kV. The TEM samples were prepared by diluting the previously mentioned samples with appropriate amounts of solvent (diluting by ten times) and then drop-casting them onto carbon-coated copper grids.

Cell Viability Assay. The cytotoxicity of the compounds was evaluated on cervical cancer cell line (HeLa cells) and non-cancerous cell line (HEK-293 cells). The cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS at 37℃, 95% humidity and 5% CO_2. Further, the assay was carried out by seeding 10000 cells/well in a 96-well plate. The cells were allowed to attach and were treated with different concentrations of the compounds for 24 h (all the dilutions were made using DMEM). After 24 h of treatment, the treated cells were added with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/mL) and were incubated for 1.5 h followed by addition of dimethyl sulfoxide (DMSO). The MTT gets reduced to formazan crystals by the live and active cells and the added DMSO dissolves these crystals. Further, the absorbance was measured at 570 nm keeping the reference filter at 630 nm using a microtiter plate reader. The viability % was calculated to by the following formula.

$$\text{Cell viability \%} = \frac{(\text{Absorbance of untreated cells} - \text{Absorbance of treated cells})}{\text{Absorbance of untreated cells}} \times 100$$

JC-1 staining. The cells were seeded at a density of 3 lakh cells/60 mm plate and were allowed to attach. These cells were further treated with the IC_{50} concentration of the compound and were incubated for 24 h. Prior to the assay, JC-1 dye was added to the cells at the concentration of 10 µM and was incubated for 30 min.Further using BD FACS (Melody) experiment was carried out. The data analysis was done on 10000 events that were gated using FlowJo software.

ROS analysis. The generation of reactive oxygen species (ROS) was analysed by seeding 10000 cells/well in a 96-well plate followed by treatment of the cells with IC_{50} concentration of the compound. During the treatment, the cells were incubated with 2′,7′-Dichlorofluorescein diacetate (DCFH-DA, 10 µM) dye, which is known to enter the cell and produce a green fluorescent colored compound di-chloro fluorescein (DCF) by reacting

with reactive oxygen.[S1,S2] The estimation of ROS was measured by the fluorescence of DCF at 535 nm. 0.1% H_2O_2 was added to the cells for the positive control.

Apoptosis: The early, late, and necrotic cell populations were identified using the FITC Annexin-V apoptosis detection kit. Following a 24 h treatment period, the cells were trypsinized, followed by a cold PBS wash and processing in accordance with the manufacturer's instructions. With the help of a BD FACS (Melody) flow cytometer, the fluorescence data were gathered in the red and green channels. Moreover, the FlowJo software was used for data analysis and fluorescence compensation on 10000 gated events.

Live/dead dual staining using Confocal Laser Scanning Microscope. The cells were seeded at a density of 80000 cells in a 35 mm plate and were allowed to attach. The cells were treated with the IC_{50} values of the compounds and were kept under incubation at 37 °C for 24 h. These treated cells were stained with dual dyes, propidium iodide (1mg/mL) for specifically staining the dead cells and acridine orange (1mg/mL) for staining the live cells. The cells were analysed using a confocal laser scanning microscope under lasers 488 nm (for acridine orange) and 514 nm (propidium iodide) for distinctly visualizing live and dead cells.

Spheroid synthesis

The 3D spheroids of Hela cells were formed by seeding nearly 20,000 cells/well in the 96-well plate. For this, the bottoms of these wells were precoated with 1.5% agarose dissolved in serum-free DMEM to prepare a nonadherent platform. This was followed by centrifugation of the 96-well plate to bring the cells to close proximity for spheroid formation at 750 rcf for 10 min. The cells were then allowed to incubate at 37 °C in a 5% CO_2 incubator for 72 h to form three-dimensional spheres.

Cytotoxicity Assay on 3D Spheroids

To determine the cytotoxicity of the moiré superlattices, spheroids, were grown in DMEM at 37 °C in the presence of 5% CO_2 in a humidified incubator. This was followed by their treatment with moiré-Zn-CuNCs and other treatment groups for 72 h. Alamar Blue assay was carried out to determine the viability of the spheroids. In this assay, a non-fluorescent resazurin dye gets reduced to a fluorescent form by the existing live cells producing a visible color change from blue to a highly fluorescent magenta color. The spectroscopic analysis was carried out by measuring the absorbance at 570 nm and in a Glomax plate reader.

Live/Dead Staining Assay of 3D Spheroids

The live/dead staining in 3D spheroids was executed by using propidium iodide and CalceinAM staining methods. The 3D spheroids were treated with the treatment groups and were allowed to incubate for 72 h. Further, the media were discarded once the incubation period was over. The spheroids were carefully washed twice with PBS to get rid of any debris. Further, the spheroids were incubated with 0.01 mg/mL of CalceinAM for staining the live cells, followed by incubation with propidium iodide (0.0 1 mg/mL) for staining the dead cells. Using a confocal laser scanning microscope the live cells and dead cells were distinguished by green and red fluorescence, respectively.

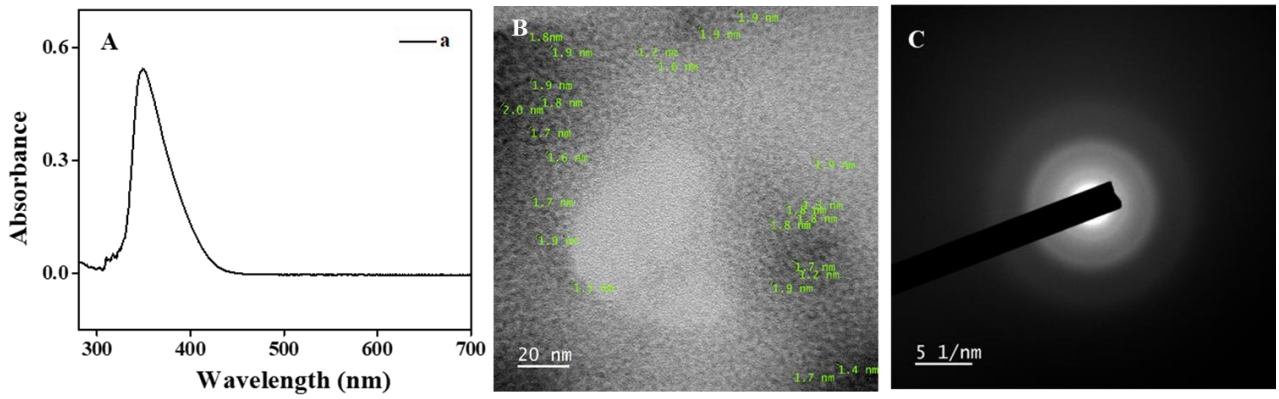

Figure S1: (**A**)(**a**) UV-Vis spectrum of the as-synthesized CuNCs. (**B**) Transmission electron microscopy image of the CuNCs and (**C**) selected area electron diffraction pattern acquired on a typical area shown in image B.

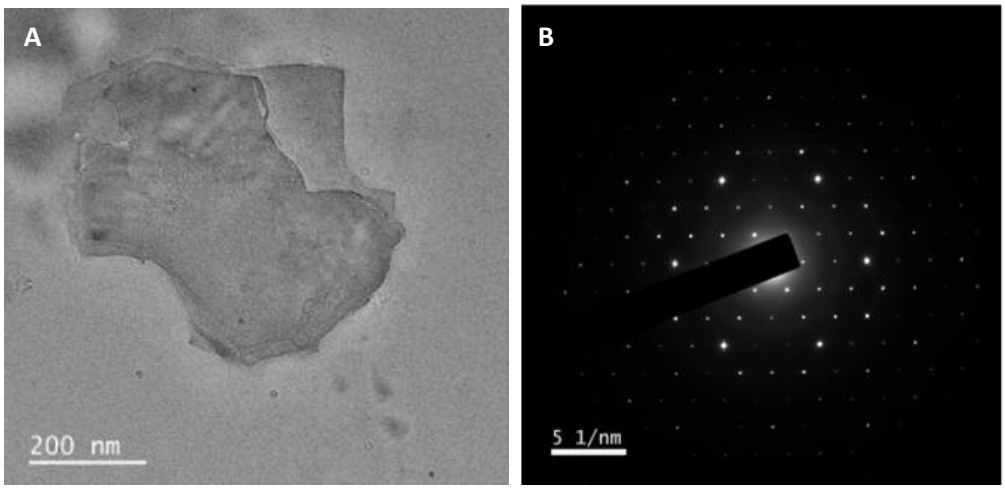

Figure S2: (**A**) Transmission electron microscopy image of Zn-CuNCs and (**B**) corresponding selected area electron diffraction pattern acquired on a typical area shown in image A.

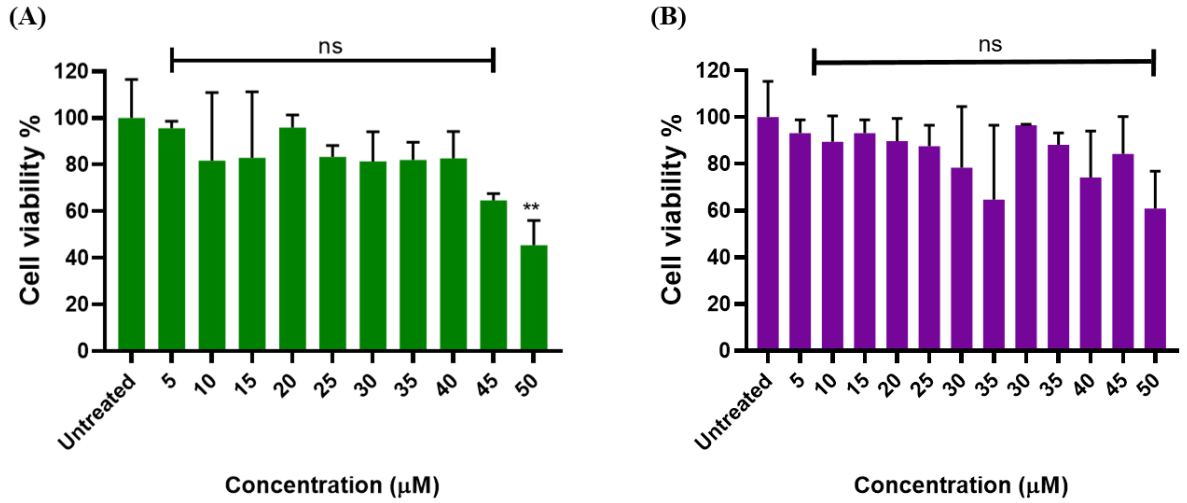

Figure S3: Cell viability results based on MTT assay of HeLa cells treated with (**A**) TPP and (**B**) Zn-TPP. (The title on the x-axis "Concentration (μM)" in all the images here represents the concentration of Cu in the respective samples for analysis.) Statistical significance is represented by ** ($p<0.01$). The values are represented as mean ± standard deviation (SD) of three individual experiments.

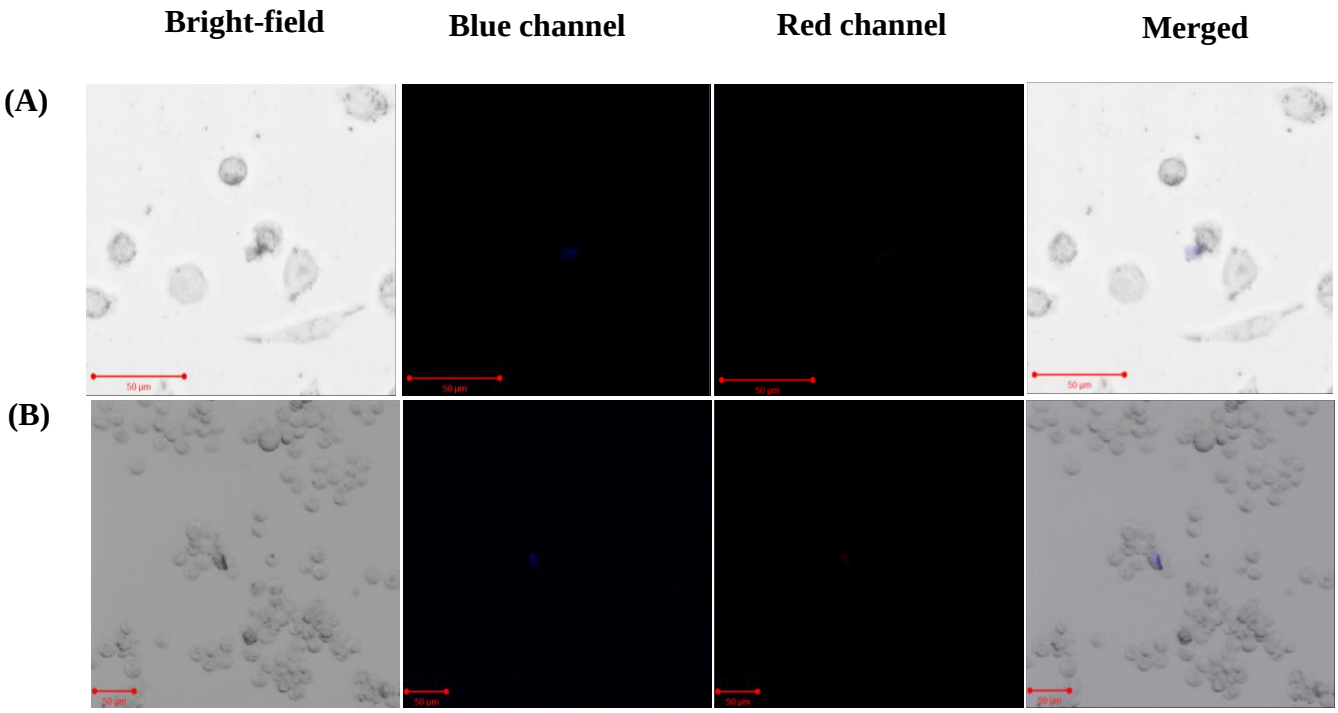

Figure S4. Confocal laser scanning microscopy images of HeLa cells treated with moiré-Zn-CuNCs at (**A**) 0 h, and (**B**) 3 h time intervals.

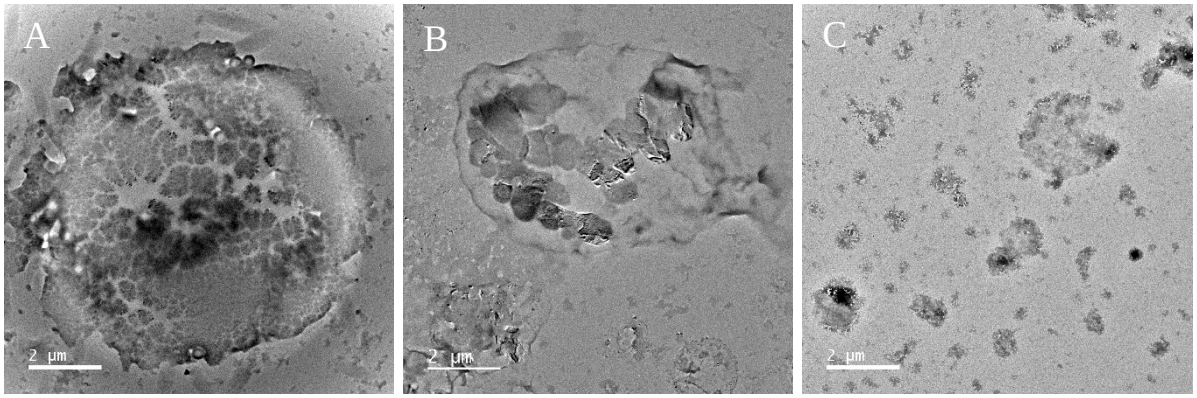

Figure S5: Transmission electron microscopy (TEM) images of (**A**) HeLa cells and those upon treatment with moiré-Zn-CuNCs for (**B**) 0 h and (**C**) 2 h.

Table S1: IC_{50} values for all compounds (used herein) as calculated from MTT assay in Figure 2 of the manuscript.

Sample	IC_{50}
CuNCs treated HeLa cells	48.7 µM
Zn-CuNCs treated HeLa cells	0.76 mM
Moiŕe-Zn-CuNCs treated HEK cells	-
Moiŕe-Zn-CuNCs treated HeLa cells	11.9 µM

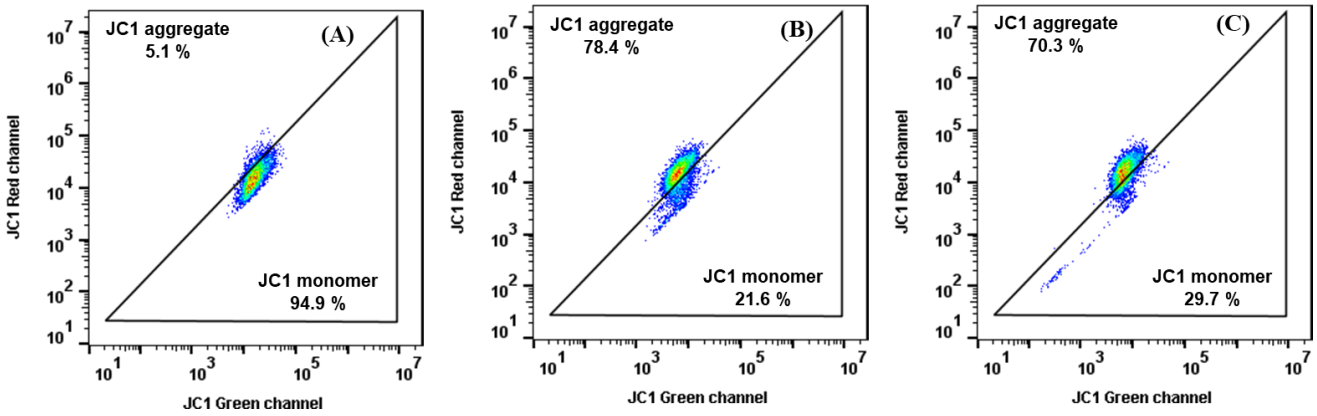

Figure S6: Typical flow cytometry diagrams of JC-1 dye stained HeLa cells treated with (**A**) CCCp (positive control), (**B**) CuNCs, and (**C**) Zn-CuNCs.

Table S2: Population percentage of JC-1 aggregate and JC-1 monomer after membrane potential analysis using JC-1 dye.

Sample	**JC-1 aggregate population %**	**JC-1 monomer population %**
Untreated	94.15 %	5.85 %
CCCp (positive control)	5.1 %	94.9 %
CuNCs	78.4 %	21.6 %
Zn-CuNCs	70.3 %	29.7 %
moiré-Zn-CuNCs	12.8 %	87.2 %

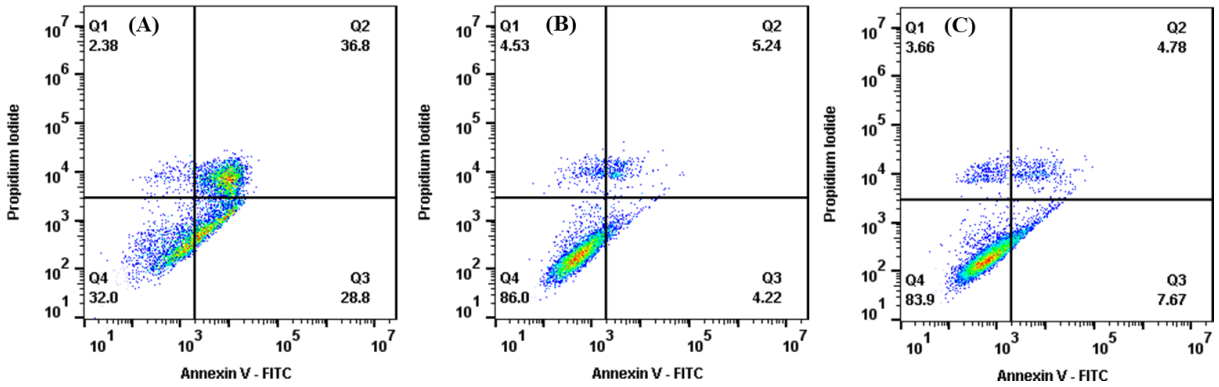

Figure S7. Typical flow cytometry diagrams of apoptotic population of HeLa cells treated with (**A**) cisplatin (positive control), (**B**) CuNCs and (**C**) Zn-CuNCs.

Table S3: Healthy cell and apoptotic cell population percentage after apoptosis assay

Sample	**Healthy population %**	**Apoptotic population %**
Untreated	88.2 %	9.4 %
Cisplatin (positive control)	32 %	65.6 %
CuNCs	86 %	9.46 %
Zn-CuNCs	84 %	12.45 %
moiré-Zn-CuNCs	59.2 %	37.4 %

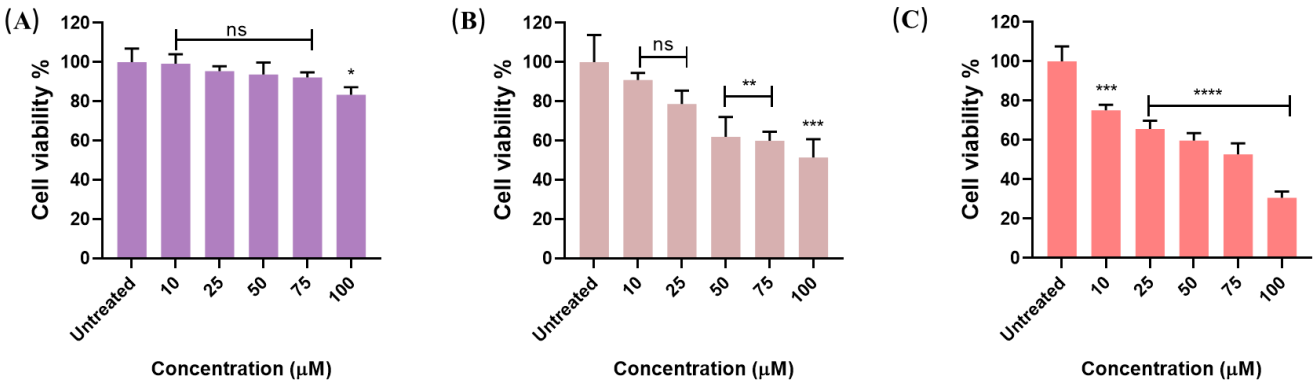

Figure S8: Cell viability results based on Alamar Blue assay of 3D spheroids of HeLa cells treated with (**A**) CuNCs ,(**B**) Zn-CuNCs, and (**C**) moiré -Zn-CuNCs. (The title on the x-axis "Concentration (μM)" in all the images here represents the concentration of Cu in the respective samples for analysis.) Statistical significance is represented by ** ($p<0.01$). The values are represented as mean ± standard deviation (SD) of three individual experiments.

References:

1. A. Pramanik, D. Laha, S. K. Dash, S. Chattopadhyay, S. Roy, D. K. Das, P. Pramanik and P. Karmakar, *Materials Science and Engineering: C*, 2016, **68**, 327-337.

2. D. Laha, A. Pramanik, S. Chattopadhyay, S. k. Dash, S. Roy, P. Pramanik and P. Karmakar, *RSC Advances*, 2015, **5**, 68169-68178.