# Electronic Supporting Information

# Atomically Precise Copper Nanoclusters Mediated Fenton-like Reaction for Cancer Chemodynamic Therapy

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## Experimental Section

### Materials and methods:

Copper sulphate pentahydrate ($CuSO_4.5H_2O$), Hydrazine Hydrate ($NH_2NH_2.H_2O$), and morpholine were acquired from SRL. Bromoacetyl bromide was purchased from TCI. 2-Mecraptobenzimidazole was procured from Avra. 3,3′,5,5′-Tetramethylbenzidine (TMB), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA), Glutathione (GSH), Fluorescein Diacetate (FDA), Propidium Iodide (PI), and 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. Antibodies were purchased from cloud clone and Bio-Rad.

The UV-vis and fluorescence emission spectra were acquired on Shimadzu UV-2600i and Fluormax-4 spectrometers, respectively. FLS spectra were recorded with the help of the FLS-1000-xs-t instrument. ATR-FTIR spectra recorded on Bruker ALPHA-II instrument. The high-resolution TEM images were acquired from a JEM 3200FS electron microscope. The solid sample of CuNCs@MorMB was prepared in Milli-Q water and drop cast on a TEM grid. To study the morphology of the nanoclusters, a scanning electron microscope (SEM) was used, and the cluster's elemental composition was determined using energy-dispersive spectroscopy (EDS) on an Apreo S LoVac. The zeta potential of the copper nanoclusters was measured using Zetasizer Nano ZS90 (Malvern Instruments). The X-ray photoelectron spectroscopy (XPS) of nanoclusters was studied using the PHI 5000 Versa Probe III instrument. Mass spectra were recorded with the help of Bruker MALDI-TOF-MS. Cell images stained with a DCFH-DA probe were captured with the help of an Olympus fluorescence microscope. Uptake studies were performed using confocal laser scanning microscope (CLSM) from Zeiss LSM880 (Airyscan). The cell viability assays were performed with the help of a SYNERGY H1 microplate reader. Thin layer chromatography was performed on Merck Kiesel gel 60, F254 plates with a layer thickness of 0.25 mm. Column chromatography was performed on silica gel (100-200 mesh) using a mixture of methanol and dichloromethane solvents as mobile phase. $^1$H NMR spectral data were collected at, 500 MHz (JEOL), $^{13}$C NMR was recorded at 125 MHz. The EPR experiments were performed at room temperature (298 K) on a Bruker Biospin EMXmicro A200 spectrometer.

### Synthesis of Ligand:

#### Synthesis of 2-Bromobenzimidazole (1)[1]

2-Mercaptobenzimidazole (5g, 33 mmol, 1 equiv.) was treated with HBr (5 mL, 45 mmol, 1.35 equiv.) in 50 mL acetic acid at 0°C, then bromine solution (6 mL, 120 mmol, 3.63 equiv.) was slowly added into it dropwise. After 10 min of stirring, the solution was transferred to a flask containing 100 mL acetic acid. A thick orange mixture was stirred for 4.5 h at 35°C and then diluted with 100 mL water. The product obtained was precipitated out by the addition sodium hydroxide pellets until the pH reaches to 4. The resulting precipitate were filtered and washed with water and dried to afford white colour product 2-Bromobenzimidazole. (Yield = 55%). $^1$H-NMR (500 MHz, DMSO-$d_6$) δ 13.23 (s, 1H), 7.51 (s, 2H), 7.20 (dd, 2H, J = 5.9, 2.9 Hz); $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 126.76, 122.33.

#### Synthesis of Mor-BAB (2)[2]

Morpholine (100 mg, 1.21 mmol, 1 equiv.) and $K_2CO_3$ (239 mg, 1.82 mmol, 1.5 equiv.) was taken in 5 mL DCM, bromo acetyl bromide (294.9 mg, 1.46 mmol, 1.20 equiv.) was slowly added at 0°C and then the mixture was stirred at 35°C for overnight. After the completion of reaction, the organic phase was extracted with water and dried over anhydrous $Na_2SO_4$. Then, the organic layer was evaporated to get colourless viscous liquid MOR-BAB. (Yield = 76%). $^1$H NMR (500 MHz, $CDCl_3$) δ 3.85 (s, 2H), 3.75–3.71 (m, 2H), 3.71–3.67 (m, 2H), 3.63 (dd, 2H, J = 8.5, 3.5 Hz), 3.54–3.49 (m, 2H); $^{13}$C NMR (126 MHz, $CDCl_3$) δ 165.41, 66.57, 66.36, 47.14, 42.40, 25.57.

**Synthesis of MorBB (3)**

Mor-BAB (180 mg, 0.86 mmol, 1 equiv.) was added into a stirring solution of 2-bromobenzimidazole (170 mg, 0.86 mmol, 1 equiv.) and $K_2CO_3$ (178 mg, 1.29 mmol, 1.5 equiv.) in DMF at 35°C. The mixture was allowed to stir for 24 h. After that, the solution was dissolved in DCM, and the organic layer was extracted with brine solution, dried over anhydrous $Na_2SO_4$. The solvent was evaporated under reduced pressure and the obtained product was purified using silica column chromatography with DCM/MeOH (98/2) as eluent to give white colour solid MorBB. (Yield =86%). $^1$H NMR (500 MHz, $CDCl_3$) δ 7.78–7.65 (m, 1H), 7.29–7.26 (m, 1H), 7.26–7.20 (m, 2H), 4.95 (s, 2H), 3.82–3.69 (m, 4H), 3.68–3.60 (m, 2H), 3.60–3.52 (m, 2H); $^{13}$C NMR (126 MHz, $CDCl_3$ ) δ 164.16, 143.51, 136.45, 130.93, 124.04, 123.35, 120.16, 109.74, 67.28, 66.85, 46.62, 45.95, 43.14.; HRMS (ESI): calculated for $[M+H]^+$ m/z = 324.0348, found: 324.0355.

**Synthesis of MorMB (4)**

MorBB (135 mg, 0.41 mmol, 1 equiv.) and thiourea (63 mg, 0.83 mmol, 2 equiv.) was dissolved in 5 mL of ethanol and refluxed for 12 h. After the reflux, solvent was evaporated under reduced pressure. The residue obtained was dissolved in DCM, and the organic phase was extracted with water, dried over anhydrous $Na_2SO_4$. The organic layer was evaporated to get the white solid compound of MorMB. (Yield = 45%). $^1$H NMR (500 MHz, DMSO-$d_6$) δ 12.82 (s, 1H), 7.29–7.24 (m, 1H), 7.22–7.14 (m, 3H), 5.20 (s, 2H), 3.73–3.67 (m, 2H), 3.67–3.62 (m, 2H), 3.61–3.56 (m, 2H), 3.48–3.41 (m, 2H); $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 169.52, 164.89, 133.88, 131.10, 123.28, 122.66, 110.58, 110.09, 66.60, 66.58, 45.38, 44.96, 42.45; HRMS (ESI): calculated for $[M+Na]^+$ m/z = 300.0783, found: 300.0785

**Synthesis of CuNCs@MorMB**

The synthesis of CuNCs@MorMB was achieved by a one-pot reaction. $CuSO_4.5H_2O$ (22.56 mg, 0.09 mmol, 1 equiv.) was treated with synthesized ligand, MorMB (25 mg, 0.09 mmol, 1 equiv.) in a 5 mL mixture of water/DMF (50:50) at 35°C. The reaction mixture turned turbid green. After 1 hour, reducing agent hydrazine hydrate (45 mg, 0.9 mmol, 10 equiv.) was added into the reaction mixture. The reaction mixture turned to pale yellow from turbid green. The stirring was continued for another 5 hours, and then the nanocluster was separated from the reaction mixture by centrifugation. The obtained precipitate was washed with water and DMF mixture to remove unreacted ligand and metal precursor, and further vacuum-dried to get a yellow-coloured precipitate of nanoclusters and stored at room temperature for further studies.

**TMB assay to detect hydroxyl radical generation**

The TMB assay was performed to check the •OH generation. Briefly, 100 μg $mL^{-1}$ CuNCs@MorMB, 100 μg $mL^{-1}$ TMB, and 40 mM hydrogen peroxide were mixed in PBS (pH 7.4 and pH 5.5). Then, the absorbance from 300 to 800 nm was measured using UV-vis spectrophotometer. The TMB solutions treated with CuNCs@MorMB or hydrogen peroxide alone were used as control groups.

**Steady state reaction kinetics**

Various concentration of $H_2O_2$ (10-50 mM) was added into a mixed solution (pH=5.5) containing CuNCs@MorMB (100 μg $mL^{-1}$) and TMB (100 μg $mL^{-1}$). Then the oxidation of TMB was monitored at λ = 650 nm. By plotting the initial velocities against $H_2O_2$ concentrations, the Michaelis–Menten kinetic curve was acquired. The rate of change in absorbance over time was used to calculate the velocity (V) for the oxidation of TMB using the Beer-Lambert law (A = εcl). Where A is the absorbance intensity at 650 nm, ε represents the molar absorption coefficient (39000 $M^{-1}$ $cm^{-1}$), l is the optical path length (0.5 cm), and c is the concentration of oxidised TMB.

The apparent kinetic parameters were measured based on the Michaelis-Menten equation:

$$V = \frac{V\max \times [S]}{K_M + [S]}$$

where [S], $K_m$, $V$, and $V_{max}$ are the concentration of hydrogen peroxide, the Michaelis-Menten constant, the initial velocity, and the maximum reaction velocity, respectively. $K_m$ and $V_{max}$ were calculated using the Lineweaver–Burk plot.

**Hydroxyl radical detection by electron paramagnetic resonance spectroscopy (EPR)**

The radical scavenger DMPO was used to trap the short-lived •OH radicals to form a stable DMPO-•OH adduct and characterized using EPR spectroscopy. A solution containing CuNCs@MorMB (100 μg/mL), $H_2O_2$ (40 mM), and DMPO (20 mM) was prepared, then the solution was transferred to a quartz capillary tube. The EPR spectrum was measured at room temperature. Similarly, EPR spectra of control experiments were performed for DMPO (20 mM), CuNCs@MorMB (100 μg/mL) + DMPO (20 mM), $H_2O_2$ (40 mM)+ DMPO (20 mM) solutions.

**GSH Depletion Assay**

DTNB (Ellman's reagent) was used to detect the GSH depletion caused by CuNCs@MorMB in the presence of $H_2O_2$. GSH (2 mM) and different concentrations of CuNCs@MorMB (6.25, 12.5, 25, 50, 100 μg $mL^{-1}$) along with $H_2O_2$ (1mM) were added in PBS (pH = 7.4) at room temperature and incubated for 30 min, then DTNB (2 mM) was sequentially added into the above solution. The readings were taken at 412 nm using a microplate reader.

**Cell culture**

Human fetal lung fibroblast cells (WI-68) and human colorectal carcinoma (HCT 116) cell were purchase from NCCS Pune, India and human liver cancer (Hep G2) cell were purchased from ATCC (American Type Culture Collection) Washington, DC, USA. Cells were cultured in DMEM media having 10% FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL) and the solution was maintained at 37 °C in an incubator with 5% $CO_2$.

**Uptake studies using CLSM:**

HCT 116 cells ($5 \times 10^4$ cells per well) were cultured and grown on cover slips for 24 h. The cells were treated with CuNCs@MorMB and incubated for 12 h. Cells were washed with PBS and coverslips were taken out from 6-well plates. Images were captured using Zeiss LSM880 (Airyscan) confocal microscope under the red channel and brightfield.

**In Vitro Cytotoxicity Assay**

The cytotoxicity of CuNCs@MorMB was evaluated using the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. First, ($10^4$ cells/well) cells were cultured in 96-well plates for 24 h. The solution of CuNCs@MorMB was prepared in DMSO (0.5% v/v) and further diluted using DMEM cell culture medium to get the working concentrations of nanoclusters. Then cells were incubated with different concentrations of CuNCs@MorMB for 72 h. After incubation for 72 h, Cells were washed twice in PBS and then incubated with 20 μL (5 mg/mL) MTT in fresh medium for 4 h at 37°C followed by solubilisation of formazan crystals in DMSO. The absorbance was recorded at 570 nm by using a microplate reader and cell viability was calculated by comparing the absorbance of treated cells relative to the untreated cells (control).

**Intracellular ROS generation**

DCFH-DA assay was performed to determine the ROS generation after the treatment with CuNCs@MorMB. Briefly, HCT 116 ($5 \times 10^4$ cells in each well) cells were exposed to CuNCs@MorMB with concentrations (50 μg $mL^{-1}$) for 3 h. Then, the cells were treated with DCFH-DA (15 μM) for 30 min, washed with PBS, and subjected for the fluorescence imaging analysis using fluorescence microscope.

Similarly, the ROS levels were quantified on HCT 116 cells ($5\times10^4$ cells in each well of 6 well plate) after treatment with CuNCs@MorMB (50 µg/mL) for 3 h, followed by treatment with DCFH-DA (15 µM) for 30 min, washed with PBS. The fluorescence emission intensities were measured with the help of microplate reader by using excitation and emission wavelengths of 485 and 535 nm, respectively. Compared to the untreated cells, the data showed the number of fold increase in ROS generation.

**Intracellular GSH detection assay**

The intracellular GSH depletion was measured with the help of the DTNB reagent. For that, briefly, $1\times10^5$ HCT 116 cells were seeded in a 6-well plate and allowed to grow in a humidified atmosphere with 5% $CO_2$ at 37 ˚C for 24 h. After that, cells were treated with CuNCs@MorMB (6.25 to 50 µg/mL) for 24 h. Furthermore, cells were lysed under ice-cold conditions, and protein was isolated. After protein isolation, the protein quantification was done with the help of the Bradford reagent, and 50 µg protein content was incubated with DTNB reagent for 30 min. After incubation, the absorbance reading was collected at 412 nm, and a comparison between the control and treated groups was plotted in the form of a bar graph.

**ICP-MS analysis**

The intracellular uptake of CuNCs@MorMB was determined by ICP-MS. HCT 116 cells were seeded to a density of approximately $1\times10^5$ cells per well in a 12-well plate. After 24 h media was removed, and cells were treated with nanoclusters (50 µg/mL) for 6 h at different temperatures (4˚C and 37˚C). After completing the incubation period with the nanoclusters, cells were washed with PBS and trypsinised. Then cells were collected by centrifugation and digested with 200 µL of nitric acid (68%) for 24 h at room temperature. After 24 h of digestion in nitric acid, the solution was diluted to a final volume of 10 mL with Milli-Q water. Then, the concentration of copper was determined using ICP-MS.

**Cell cycle arrest**

HCT 116 cells ($6\times10^4$ cells per well) were seeded in 6 well plate and cultured for 24 h. Then, cells were incubated with CuNCs@MorMB (50 µg/mL) for 24 h. After 24 h, cells were washed with PBS and trypsinised to obtain the pellet. Then, the pellets were washed with ice-cold PBS, vortexing the pellet with dropwise addition of 70% ethanol and further incubated for 2 h at 4°C to fix the cells. After this, RNAse (100 µg/mL) and PI (50 µg/mL) were added, and flow cytometric analysis was performed.

**Cell apoptosis assay**

HCT 116 cells were seeded into 6 well plate at a density of $1\times10^5$ cells per well. Cells were treated with CuNCs@MorMB for 24 h. After the incubation the cells were harvested and washed twice with ice cold PBS, followed by annexin V FITC/PI staining in annexin binding buffer for 15 min in the dark condition at room temperature and analysed by using flow cytometer.

**Estimation of Caspase-3 and Bcl-2 proteins**

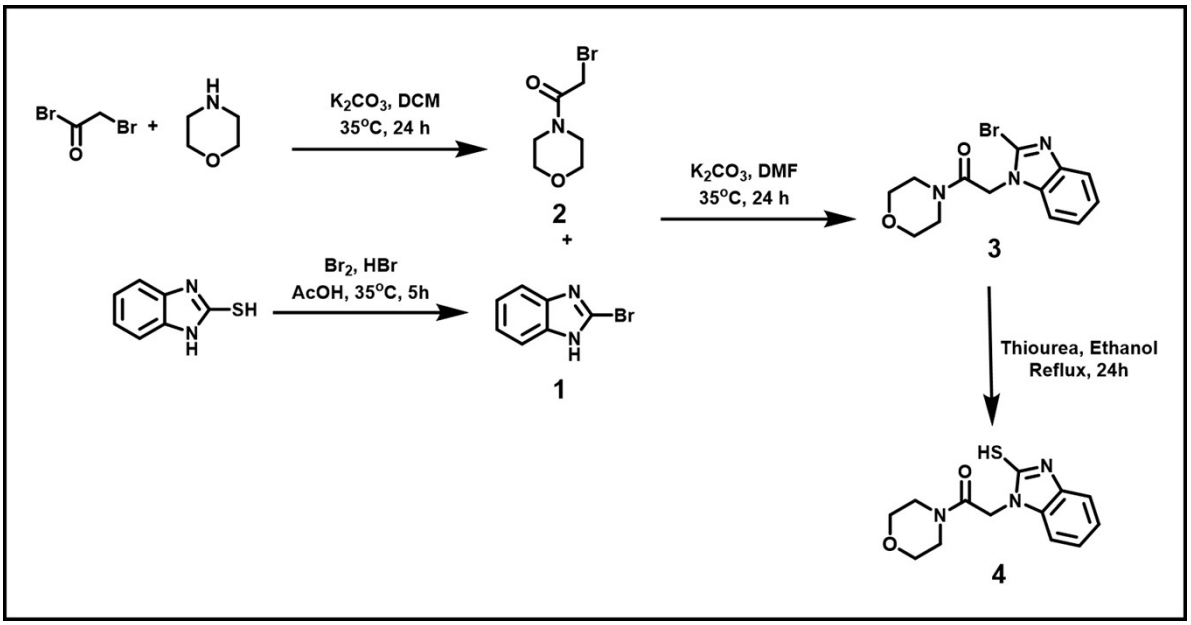
$1\times10^5$ HCT 116 cells were seeded into 6 well plates for 24 h under a humidified atmosphere with 5 % carbon dioxide at 37 C. After that, cells were incubated with CuNCs@MorMB (50 µg $mL^{-1}$) for 24 h. Furthermore, the cells were harvested using trypsin and lysed under ice-cold conditions using RIPA (Radio-immunoprecipitation assay) buffer containing 1 mM phenylmethyl sulfonyl fluoride (PMSF), a protease inhibitor. After lysis, the samples were centrifuged for 8 min at 4°C. The protein content was collected from the supernatant, and concentration was determined with the help of the Bradford reagent. Sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis was performed by loading equal amounts of protein per lane. The gels were transferred to PVDF (polyvinylidene difluoride) membranes and blocked with skim milk in PBST buffer for 1 h. Then, the membranes were washed with PBST (4 times) and incubated with primary antibodies in PBST overnight at 4 °C. After complete incubation, membranes were washed (4 times) with PBST. Subsequently, the membranes were incubated with

secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature and washed four times with PBST. The blots were visualized using protein bands and detected using enhanced chemiluminescence (ECL) reagents with the help of the Bio-Rad ChemiDoc system.

**Spheroid growth, viability, and morphology**

HCT 116 cells ($2.5\times10^4$cells per well) were seeded in an ultra-low attachment 96-well plate (U-shaped wells) and grown for 72 h to get the desired 3D spheroids. Then, spheroids were incubated with CuNCs@MorMB (50 µg/mL) for 24 h. After 24 h, spheroids were washed with 1X PBS, and further images were captured by an Olympus fluorescence inverted microscope under a bright channel. To check the viability, after 72 h incubation with CuNCs@MorMB, 3D spheroids were washed with PBS and stained with FDA/PI solution for 10 min. Then it was washed with PBS buffer twice and the images were captured using fluorescence microscope.

Similarly, spheroids were incubated with CuNCs@MorMB (50 µg/mL) for 72 h, followed by removal of medium and subsequent washing with PBS, spheroids were incubated with resazurin (50 µM) for 12 h, and viability was measured as result of resorufin fluorescence intensity at 590 nm measured by microplate reader by using excitation wavelength of 530 nm.




**Scheme S1: Synthetic route to prepare ligand MorMB**

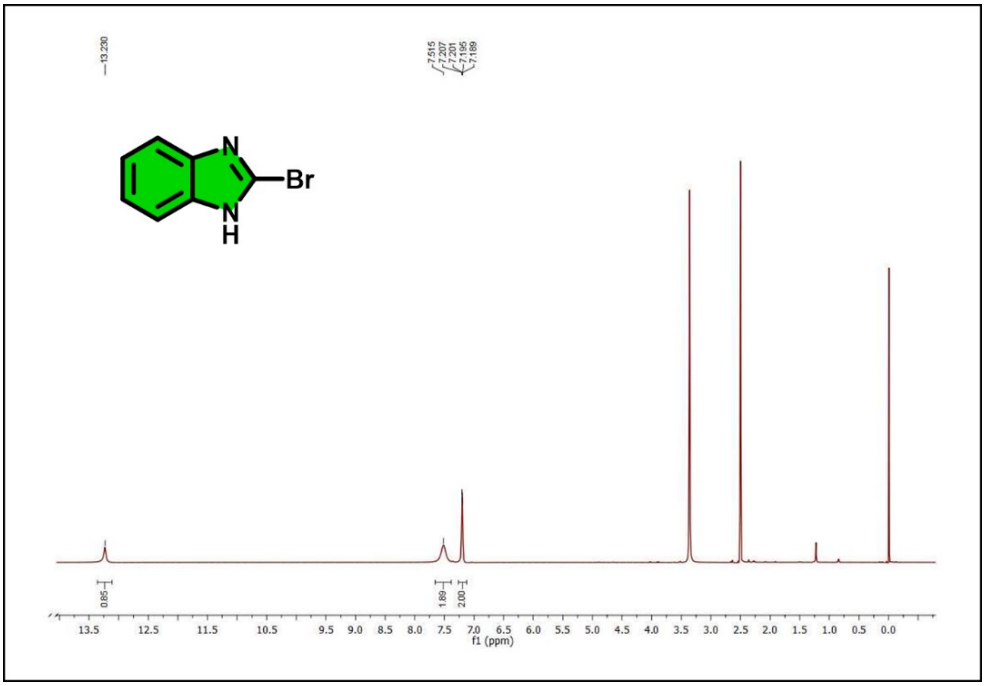



**Fig. S1: $^{1}$H NMR spectra of 2 bromobenzimidazole (1) (DMSO-d$_6$)**

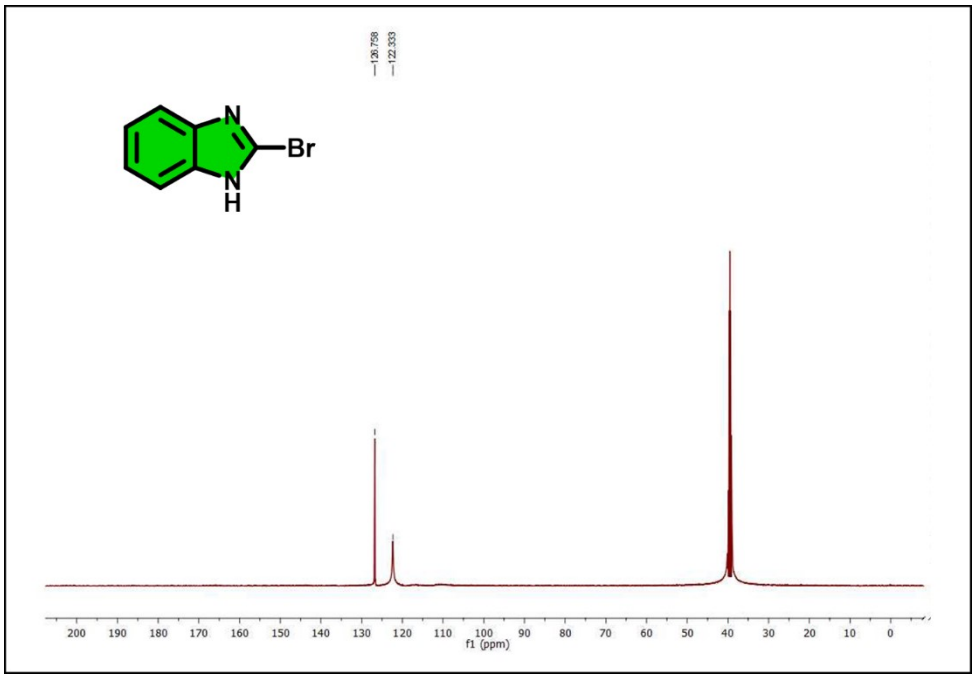



**Fig. S2: $^{13}$C NMR spectra of 2 bromobenzimidazole (1) (DMSO-d$_6$)**

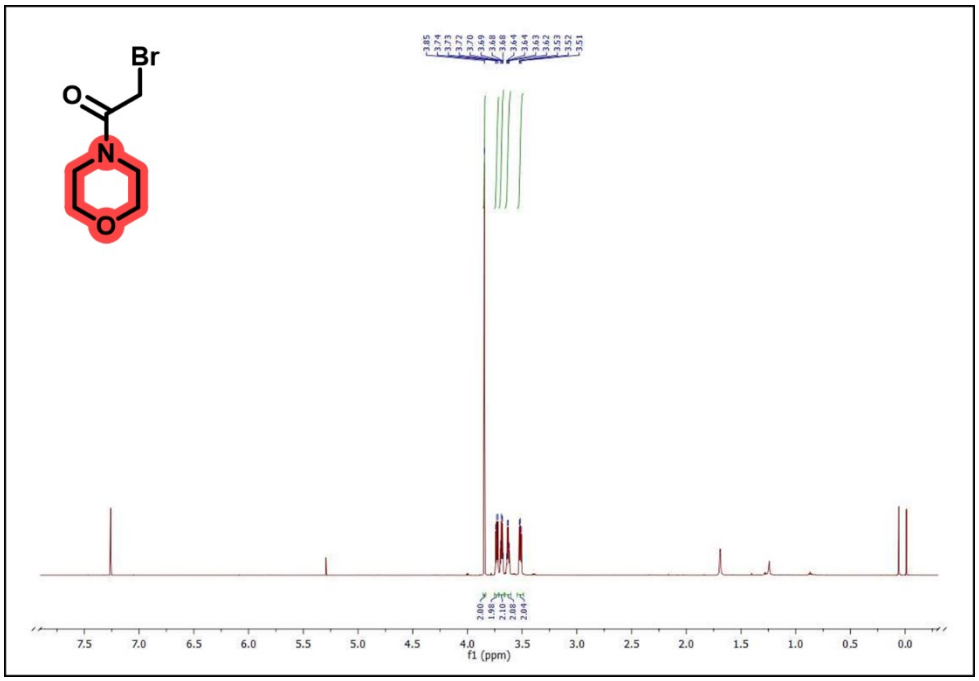



**Fig. S3: $^{1}$H NMR spectra of Mor-BAB (2) ($CDCl_3$)**

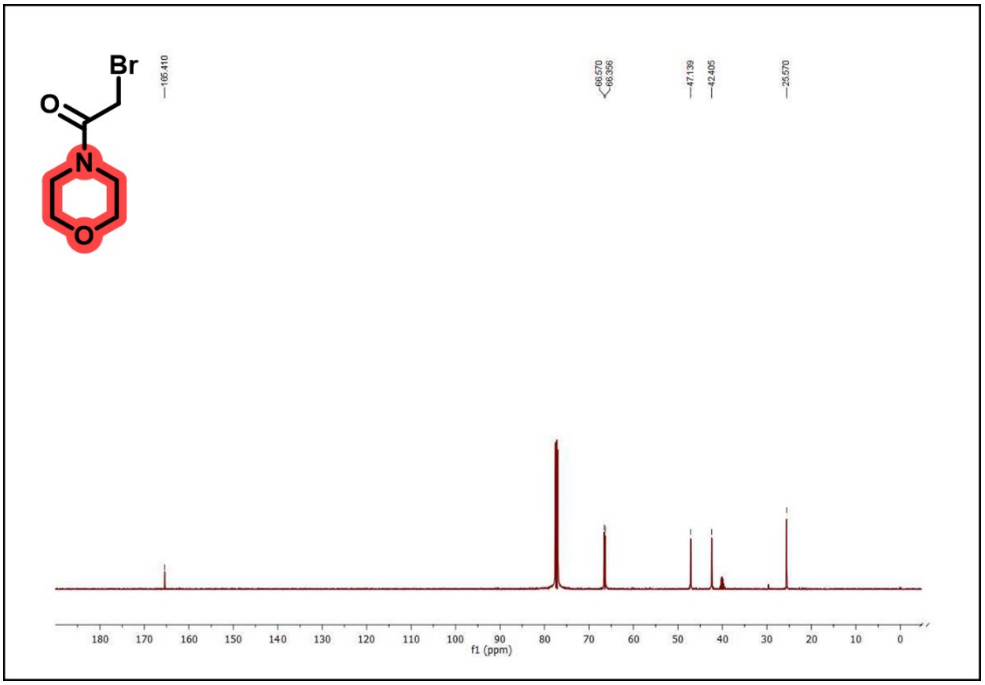



**Fig. S4: $^{13}$C NMR spectra of Mor-BAB (2) ($CDCl_3$)**

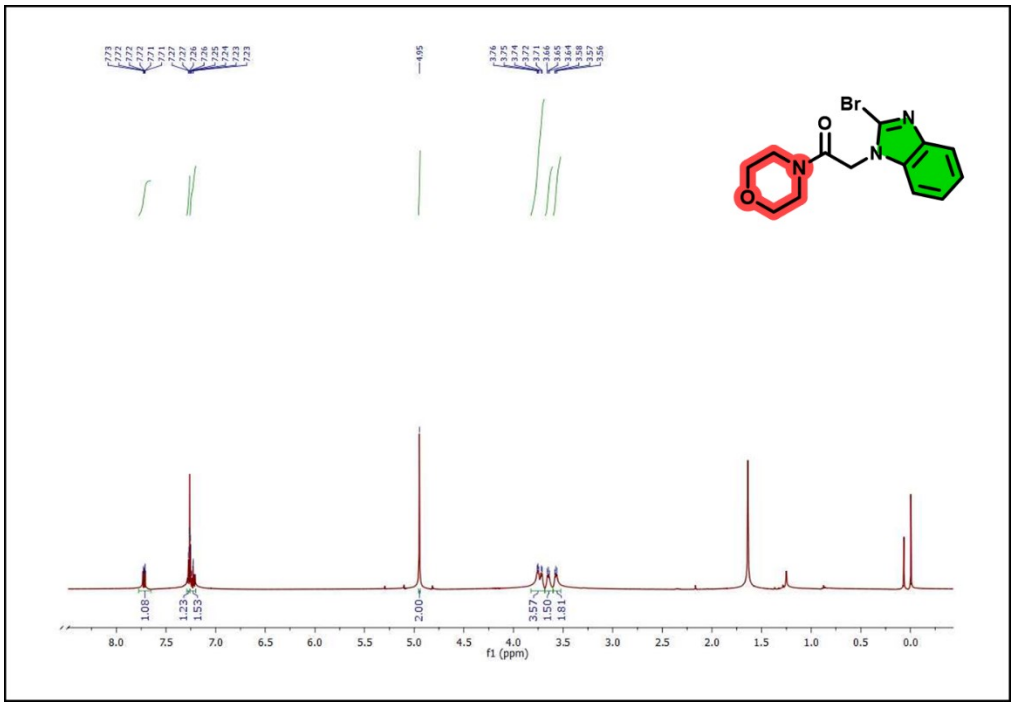



**Fig. S5: $^{1}$H NMR spectra of MorBB (3) ($CDCl_3$)**

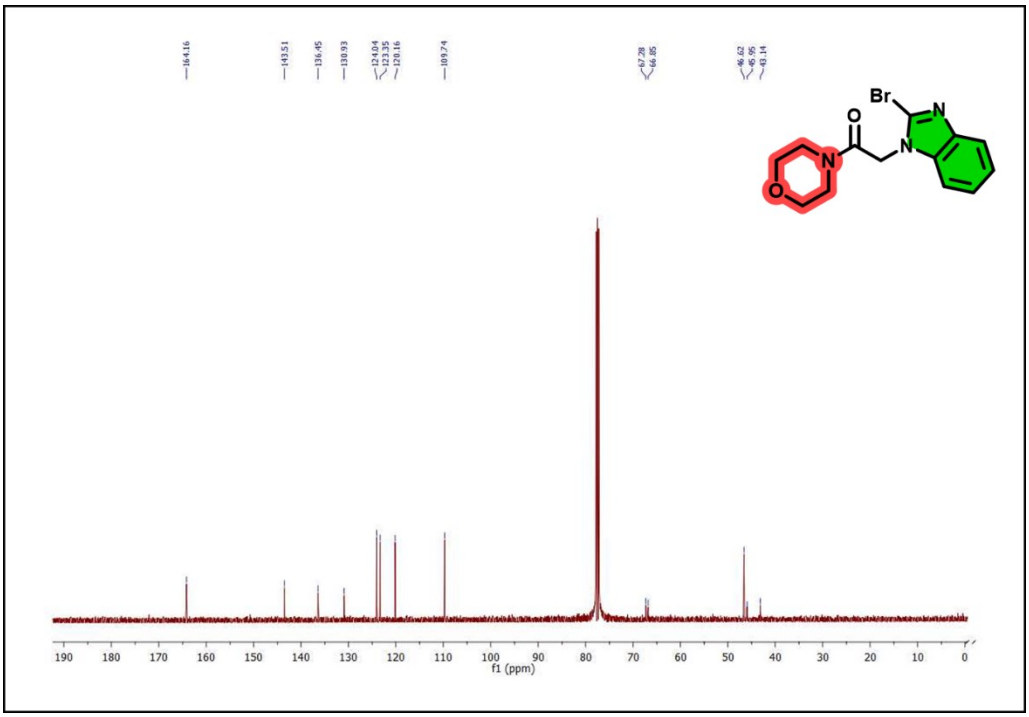



**Fig. S6: $^{13}$C NMR spectra of MorBB (3) ($CDCl_3$)**

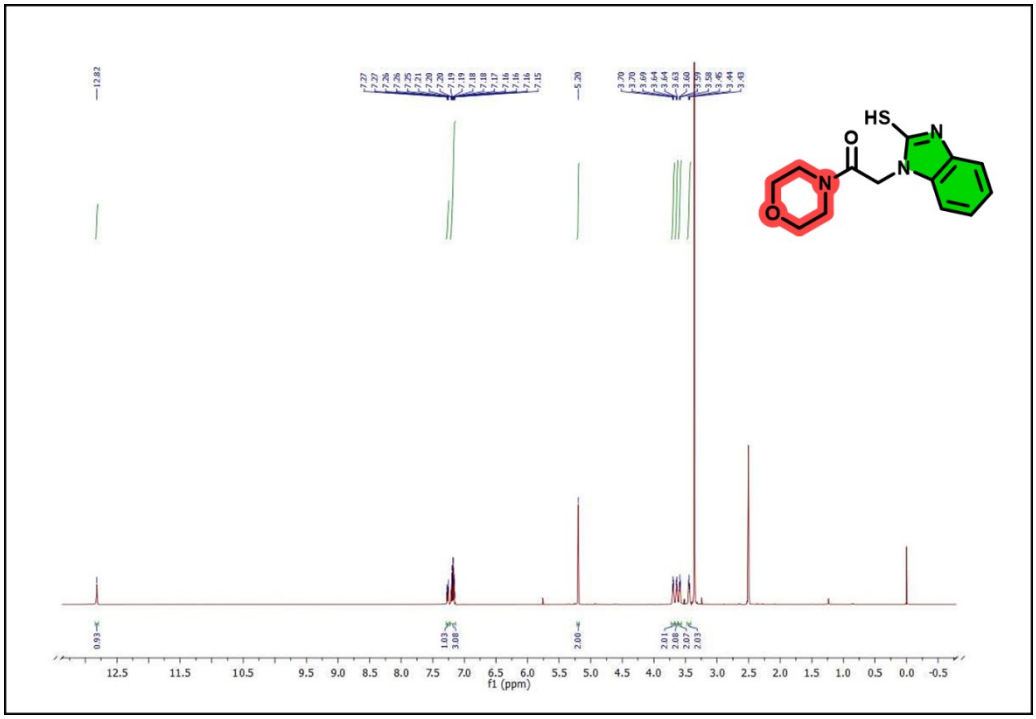



**Fig. S7: $^{1}$H NMR spectra of MorMB (4) (DMSO-$d_6$)**

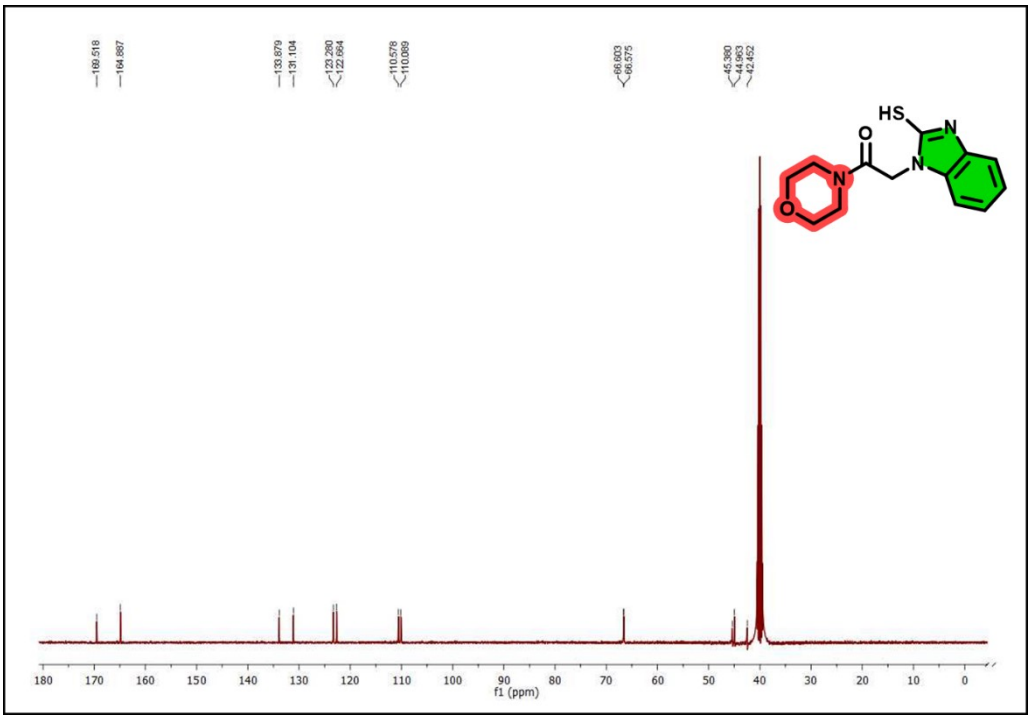



**Fig. S8: $^{13}$C NMR spectra of MorMB (4) (DMSO-$d_6$)**

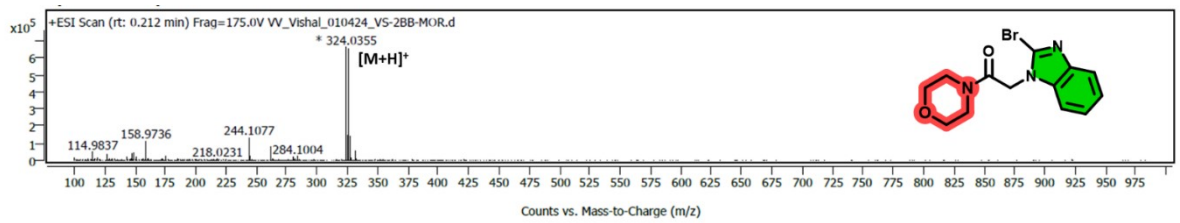



**Fig. S9: HRMS of MorBB(3)**

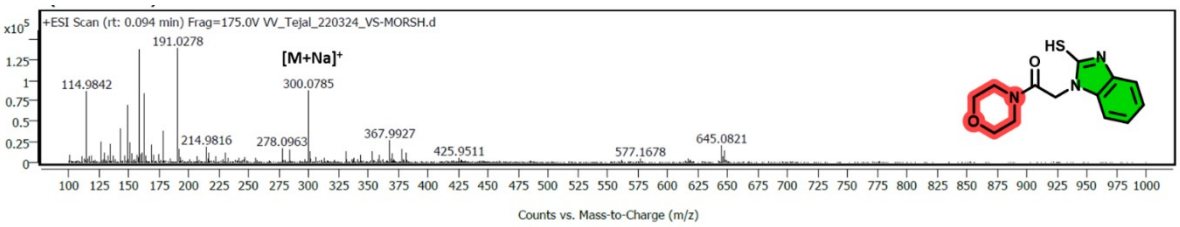



**Fig. S10: HRMS of MorMB (4)**

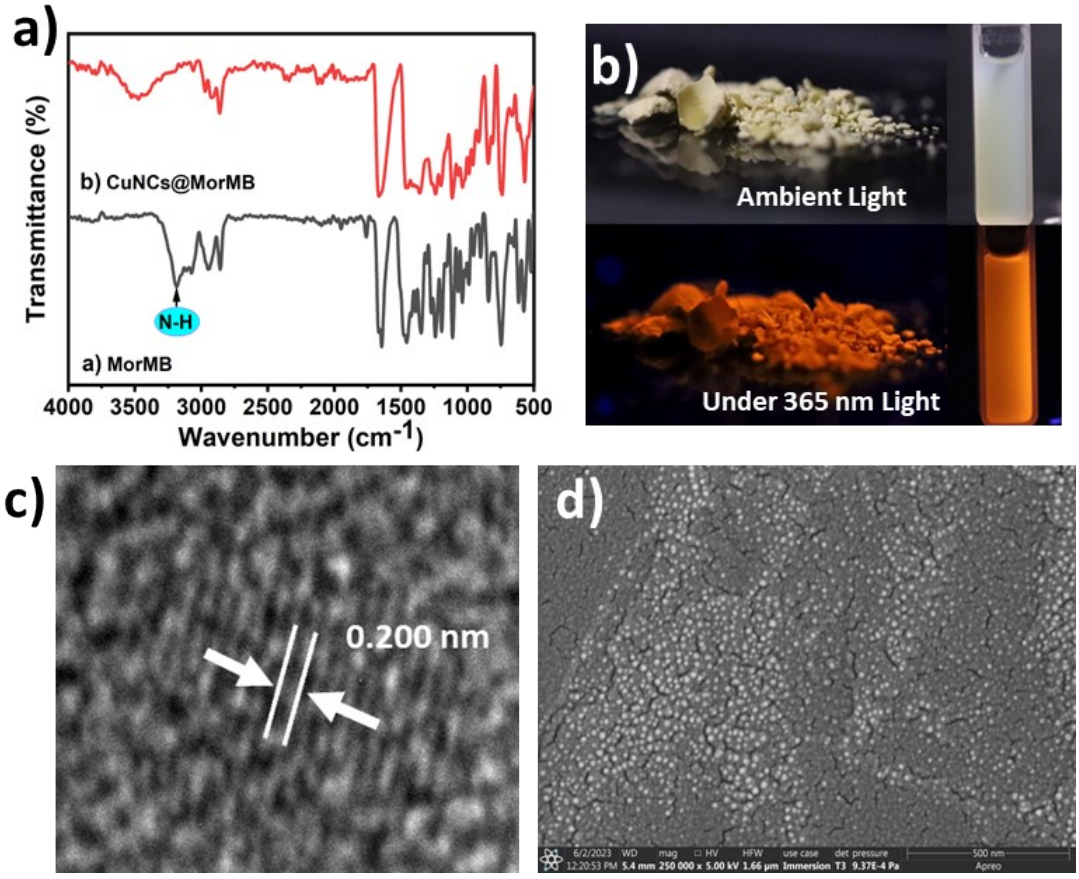



**Fig. S11: a) ATR-FTIR spectra of MorMB and CuNCs@MorMB; b) Captured images of CuNCs@MorMB in solid and liquid states under ambient light and 365 nm light, respectively; c) HRTEM image showing the interplanar distance in the nanoclusters; d) FE-SEM images of nanoclusters.**

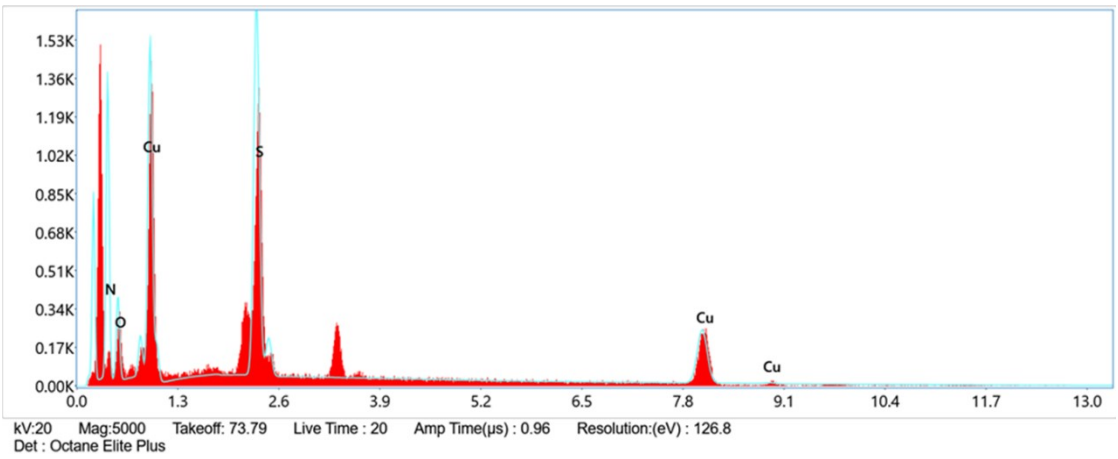



**Fig. S12: EDX analysis of CuNCs@MorMB**

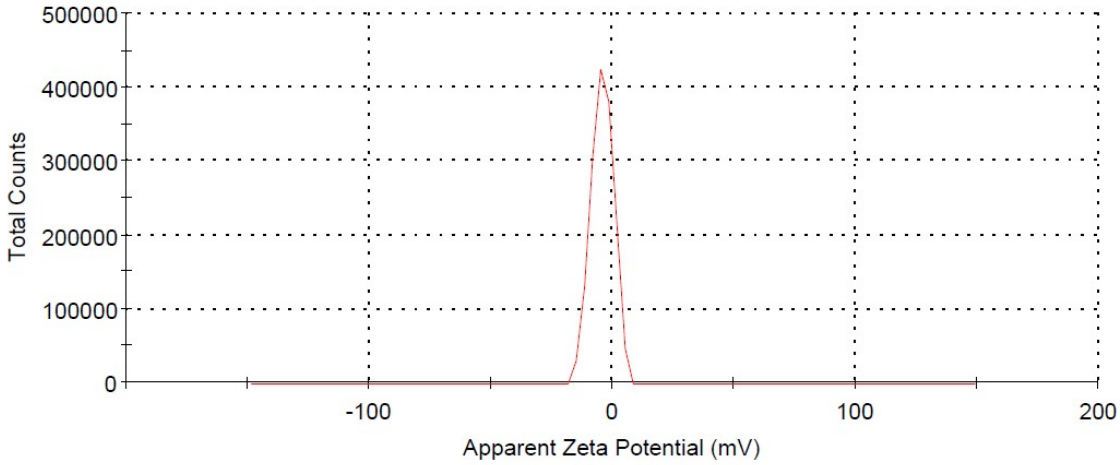



**Fig. S13: Zeta potential of CuNCs@MorMB**

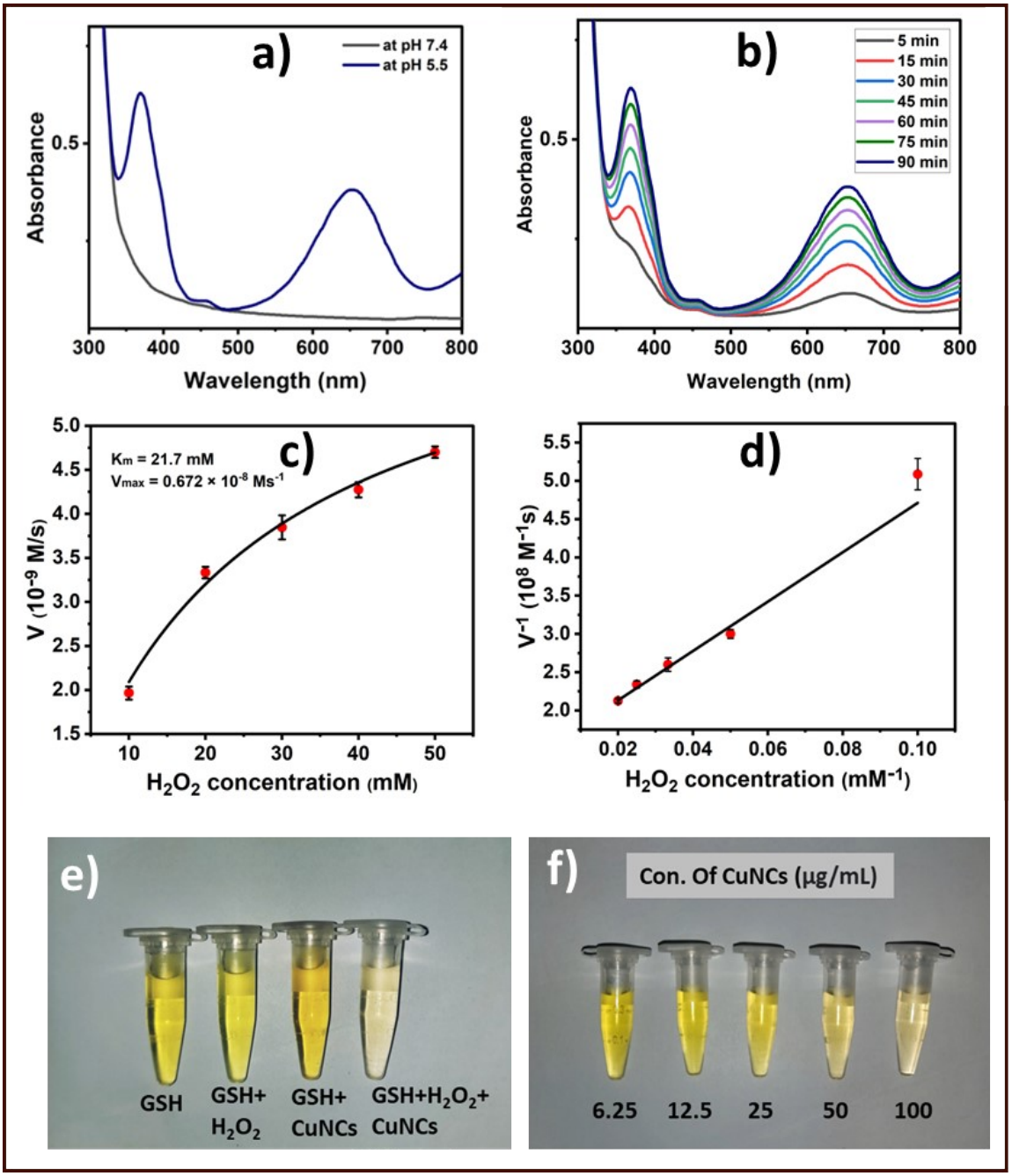
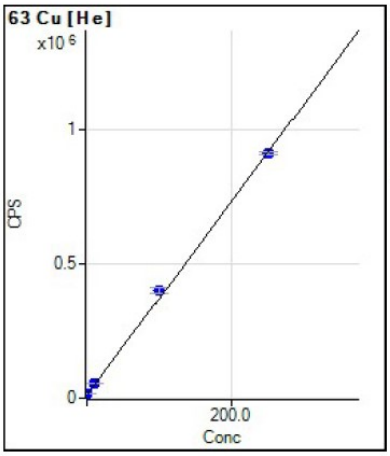



**Fig. S14: a) and b) pH and Time dependent change in oxidation of TMB during Fenton-like reaction; c) and d) Steady-state kinetic study using the Michaelis–Menten model and Lineweaver–Burk model for CuNCs@MorMB by varying the concentration of $H_2O_2$ with a constant concentration of TMB; e) and f) Visual representation of GSH depletion at different conditions and concentration of CuNCs@MorMB.**




| | Rjct | Conc. | Calc Conc. | CPS | Ratio | Det. | RSD | %RE |
|---|---|---|---|---|---|---|---|---|
| 1 | ☐ | 0.000 | 0.000 | 16012.77 | | P | 3.1 | |
| 2 | ☐ | 10.000 | 10.643 | 54508.84 | | P | 3.6 | 6.4 |
| 3 | ☐ | 100.000 | 106.378 | 400787.26 | | P | 4.3 | 6.4 |
| 4 | ☐ | 250.000 | 247.423 | 910959.21 | | P | 1.2 | -1.0 |

y = 3617.0664 * x + 16012.7667
R = 0.9995
%RSE = 9.1
DL = 0.4145
BEC = 4.427
Weight: <None>
Min Conc: 0

**Fig. S15: The standard calibration curve for Cu-63.**

| **Sample Name** | **Cu concentration at 37˚C/$10^5$ HCT 116 cells** | **Cu concentration at 4˚C/$10^5$ HCT 116 cells** |
|---|---|---|
| **CuNCs@MorMB** | 607.674 ppb | 452.848 ppb |

**Table S1: Cellular uptake (ICP-MS data) of CuNCs@MorMB at 4˚C and 37˚C after 6 h of incubation with CuNCs@MorMB.**

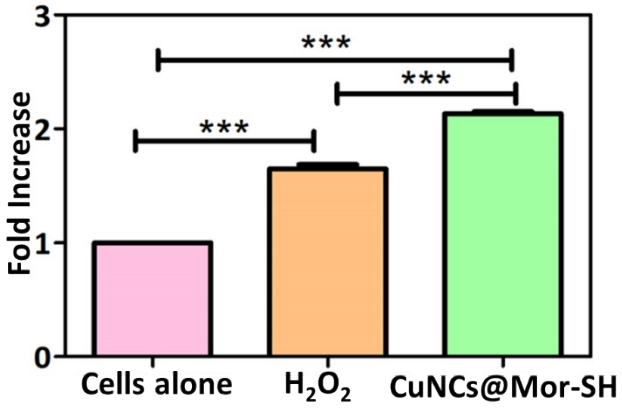



**Fig. S16: Quantitative estimation of ROS levels in CuNCs@MorMB treated HCT 116 cells and control cells.**

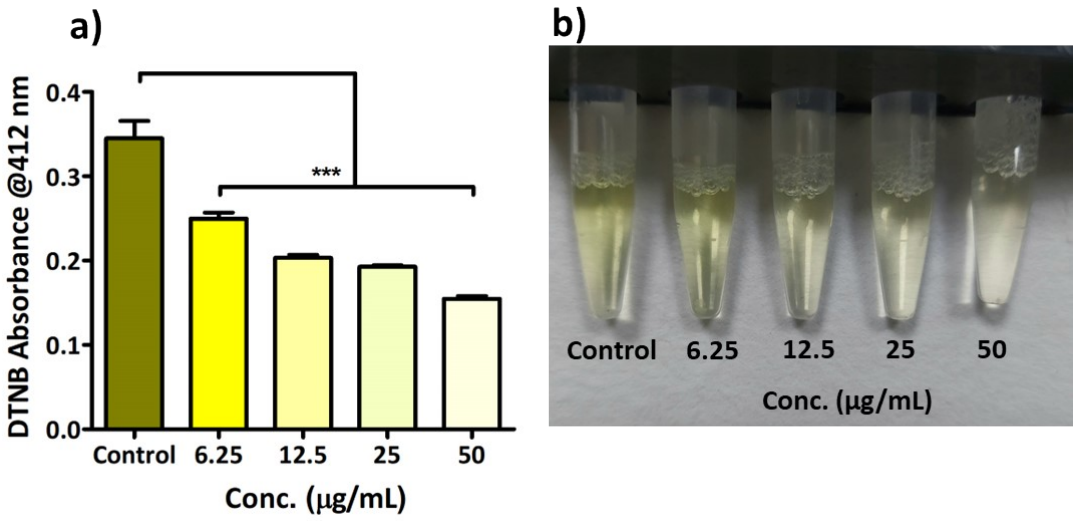



**Fig. S17: a) Intracellular GSH levels in HCT 116 cells treated with different concentrations of CuNCs@MorMB. Data represented as mean ± SD (n=3), P value was calculated using one-way NOVA with Tukey multiple comparison test; ***P<0.001 and b) Their corresponding visual representation of GSH depletion.**

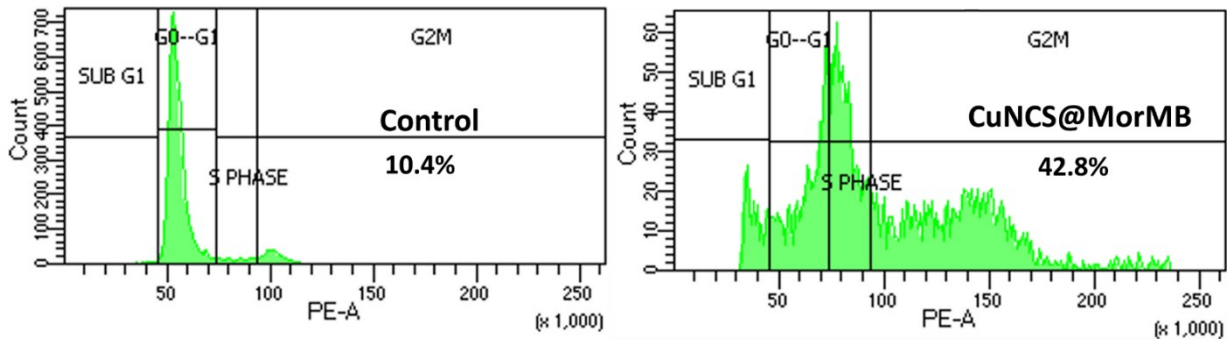



**Fig. S18: Flow cytometric analysis data of cell cycle arrest studies with PI staining in HCT 116 cells before and after treatment with CuNCs@MorMB for 24 h.**

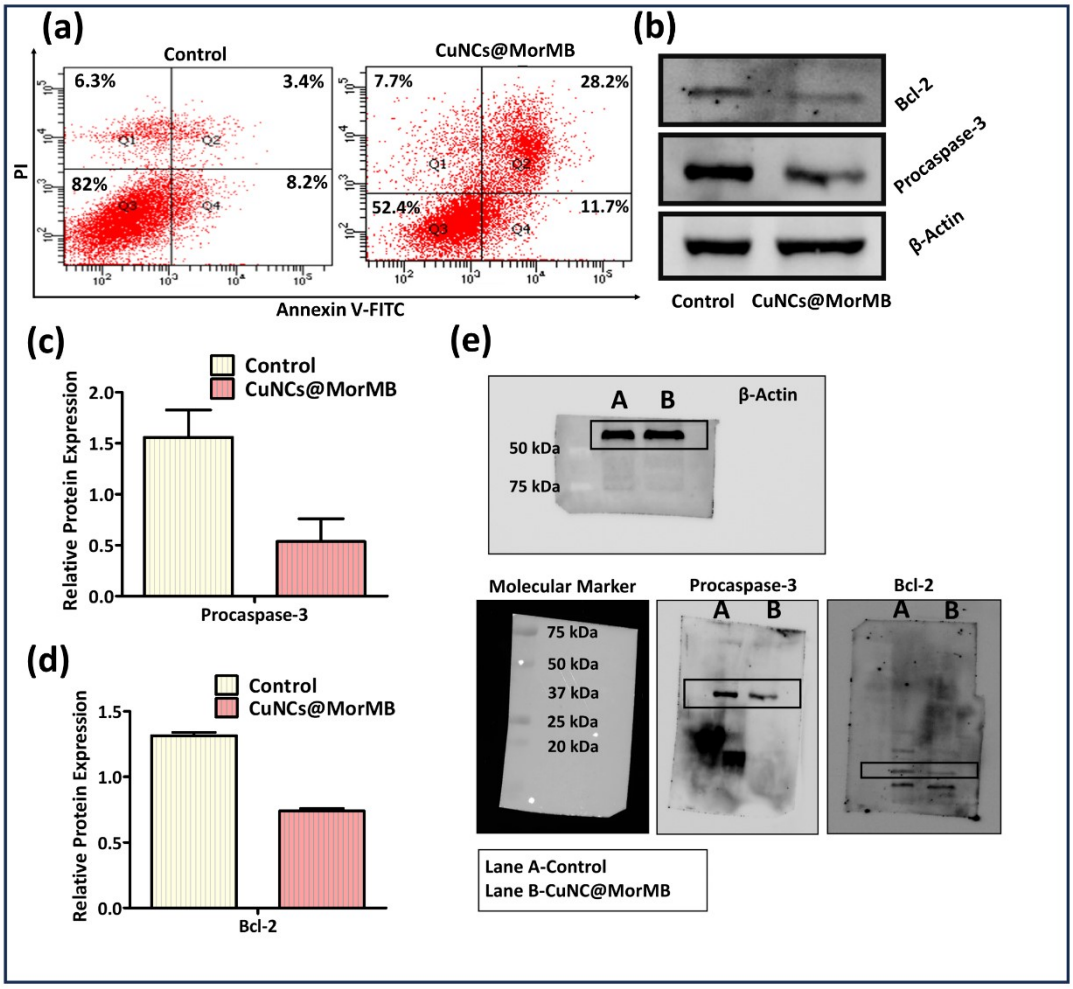



**Fig. S19: a) Flow cytometric apoptosis analysis of Annexin V-FITC/PI-stained HCT 116 cells before and after treatment with CuNCs@MorMB for 24 h; b) Estimation of Bcl-2 depletion and Caspase-3 activation with the help of Western blotting analysis; c) & d) their corresponding densitometric plots; e) Raw Western blot images as acquired from Gel Doc system.**

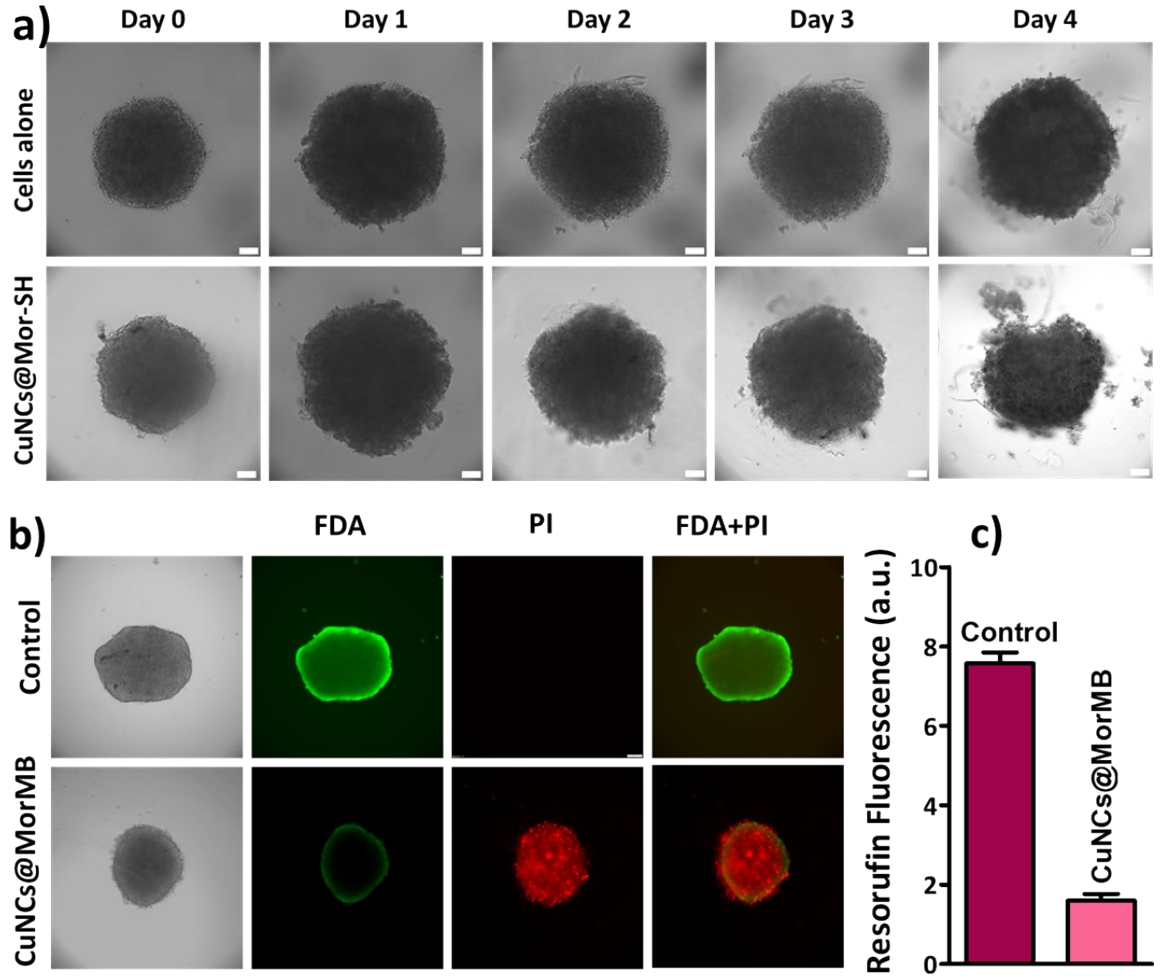



**Fig. S20: a) Morphology changes in 3D spheroids of HCT 116 cells before and after treatment with CuNCs@MorMB; scale bar: 100 µm; b) Live–dead stained fluorescence imaging of HCT 116 cell spheroids with and without CuNCs@MorMB; scale bar: 100 µm; c) AlamarBlue assay representing reduction in the cell viability of 3D spheroids after treatment with CuNCs@MorMB;**

**References:**

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