Supporting Information

Killing Cancer Cells by Delivering Nanoreactor for Inhibition of Catalase and Catalytically Enhancing Intracellular Levels of ROS

Ranjith Kumar Kankala[1], Yaswanth Kuthati[1], Chen-Lun Liu[1], Chung-Yuan Mou[2] and Chia-Hung Lee[1,*]

[1]Department of Life Science and Institute of Biotechnology, National Dong Hwa University, Hualien, 974 (Taiwan).

[2]Department of Chemistry, National Taiwan University, Taipei 106 (Taiwan).

Correspondence: Chia-Hung Lee,

Department of Life Science and Institute of Biotechnology, National Dong Hwa University, Hualien, 974 (Taiwan).

Tel: +886-3-863-3-3677

E-mail: chlee016@mail.ndhu.edu.tw

Table S1. Physical parameters related to BET, DLS, and Zeta potential of Cu-MSN and its further functionalized products.

Sample	BET Surface Area (m^2/g)	Pore Volume (cm^3/g)	Pore Size (nm)	Particle Size (nm)	Zeta Potential (mV)
Pure MSN	1586	1.83	2.5	190	-22.9±0.2
Cu-MSN	1189	1.86	2.5	169	-9.5±0.1
Cu-MSN-AT	20	0.15	N.D.	320	-22.5±0.2
Cu-MSN-AT-(calcined)	1084	1.01	1.8	N.D.	N.D.

* N.D.- Not Determined

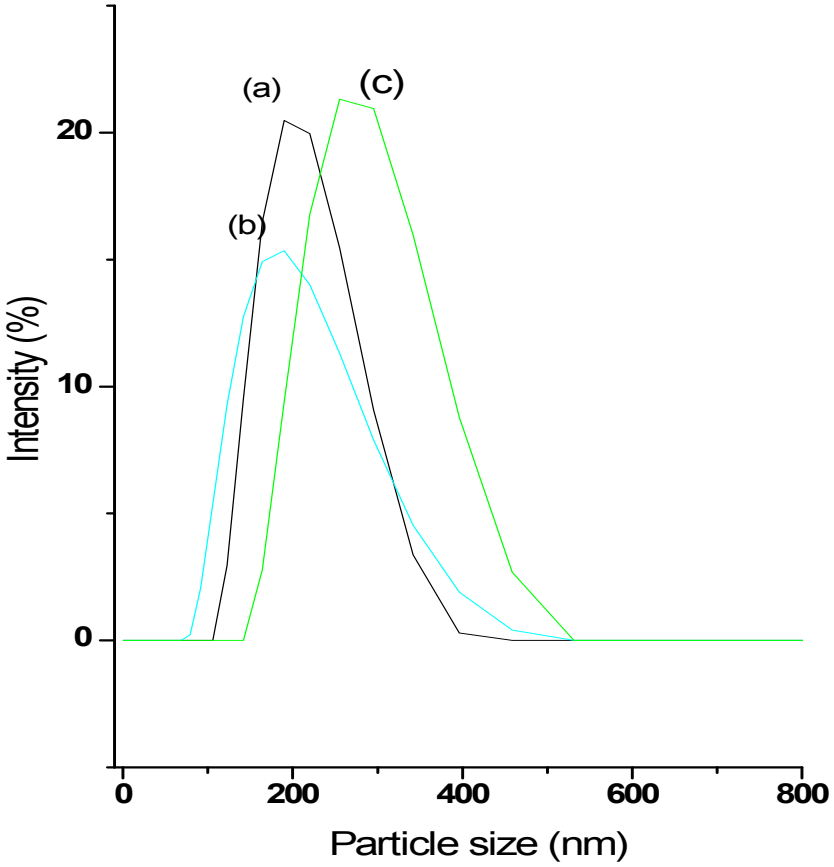

Fig. S1 Dynamic light scattering (DLS) measurement of (a) Pure MSN, (b) Cu-MSN and (c) Cu-MSN-AT.

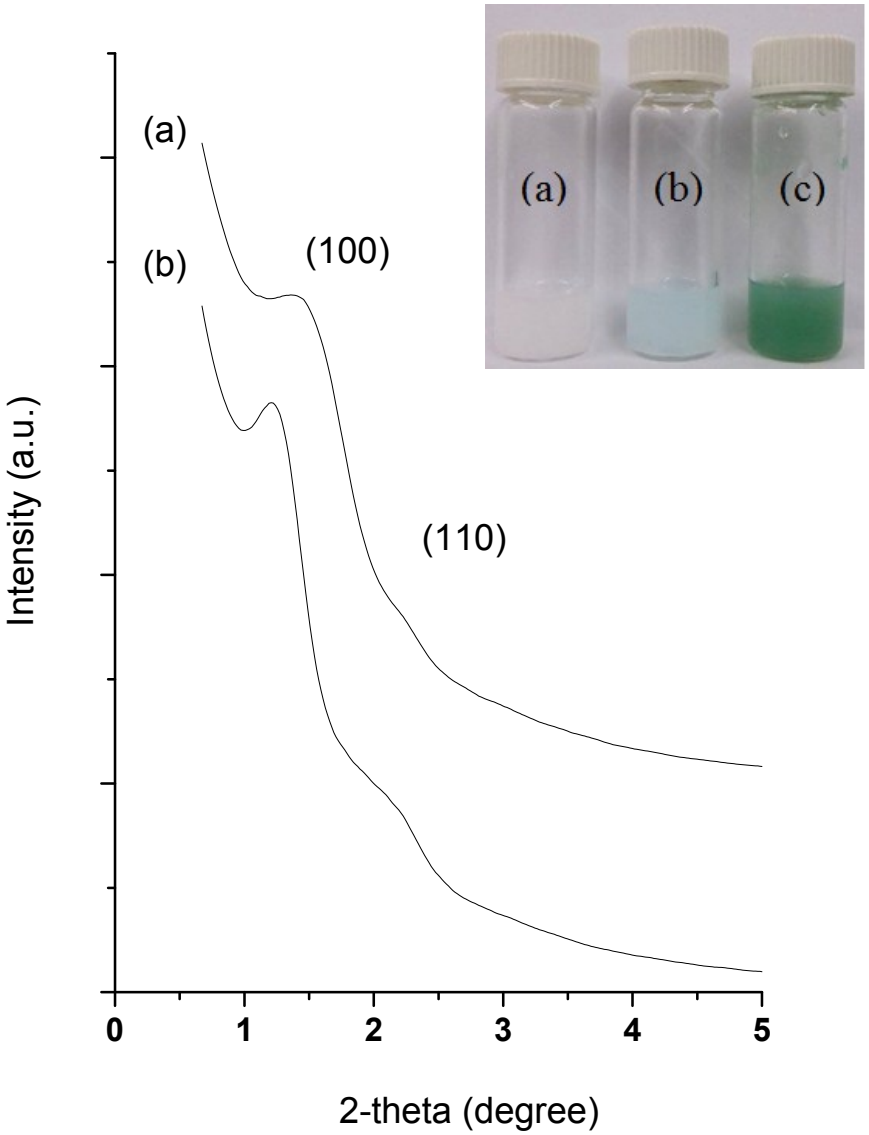

Fig. S2 PXRD images of (a) Cu-MSN and (b) Cu-MSN-AT. The inset Fig. represented the changes of the sample colour of (a) pure MSN, (b) Cu-MSN and (c) Cu-MSN-AT samples.

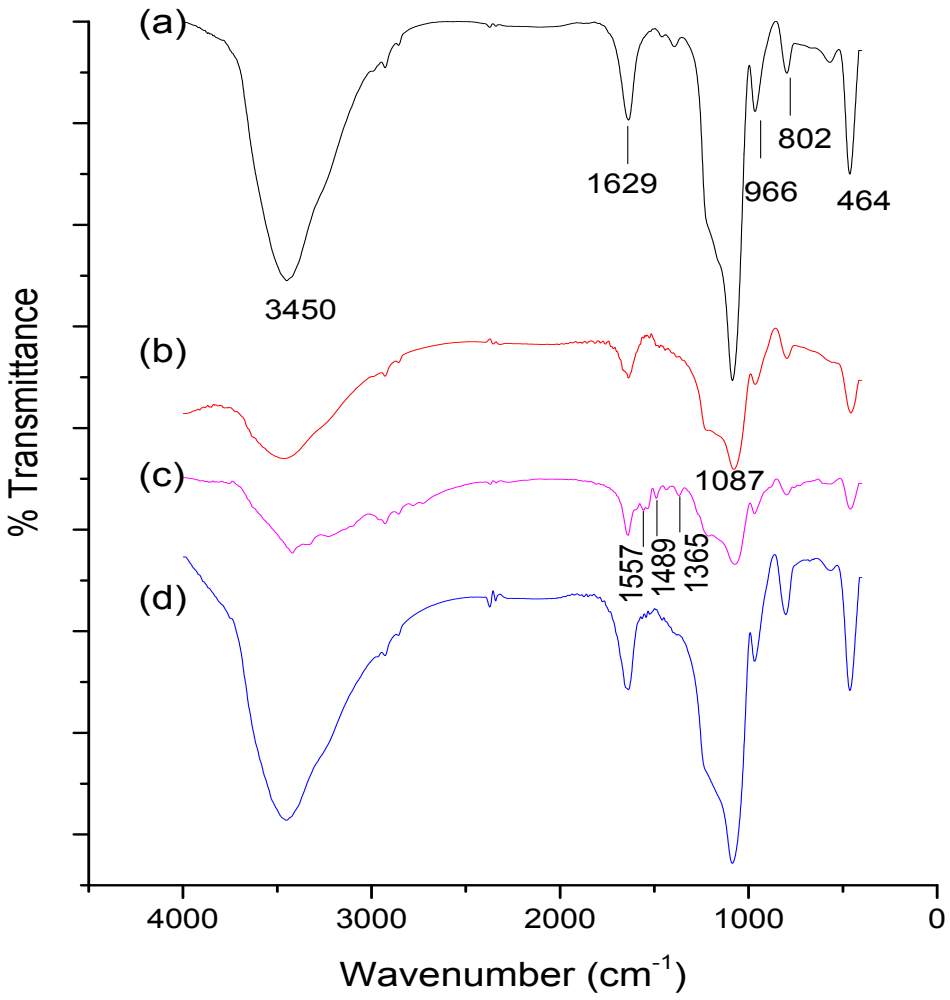

Fig. S3 FT-IR spectra of (a) Pure MSN, (b) Cu-MSN, (c) Cu-MSN-AT and (d) Cu-MSN-AT after calcination.

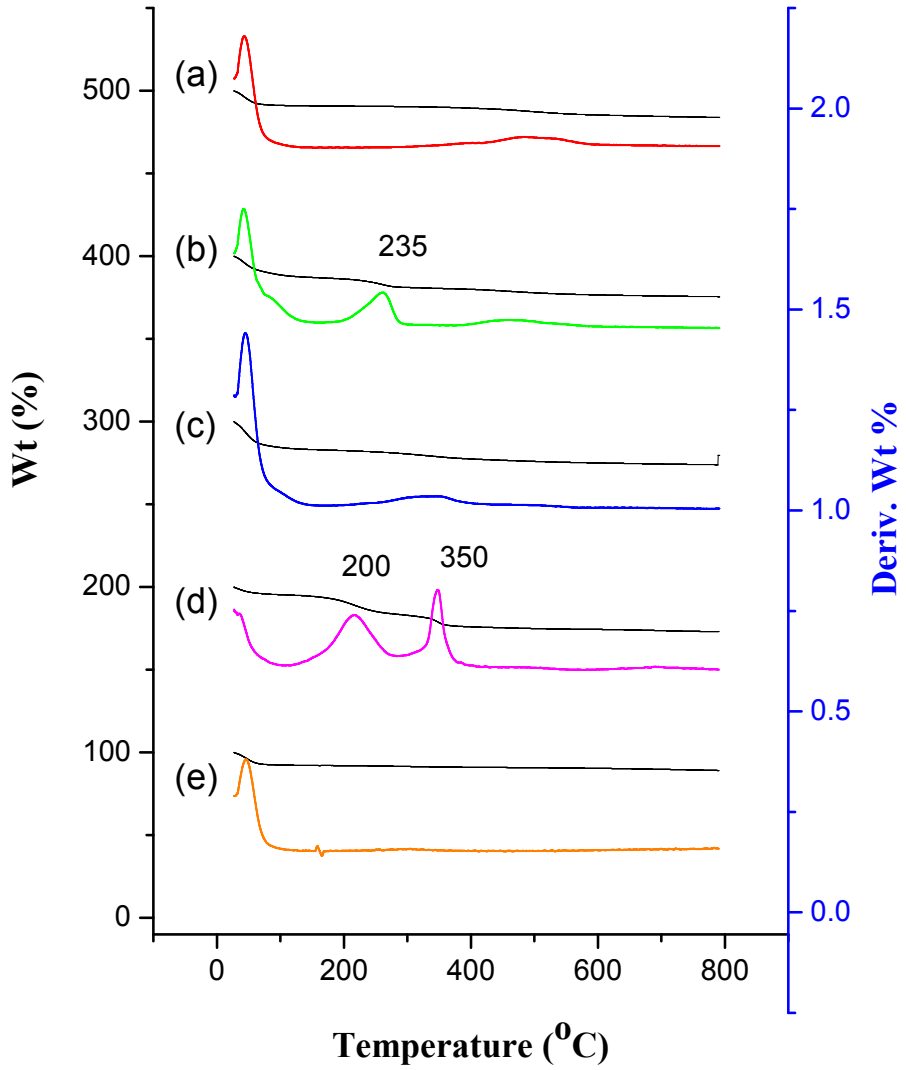

Fig. S4 TGA curves of (a) Pure MSN, (b) MSN-AT, (c) Cu-MSN, (d) Cu-MSN-AT and (e) Cu-MSN-AT after calcination. Coloured curves are differential curves.

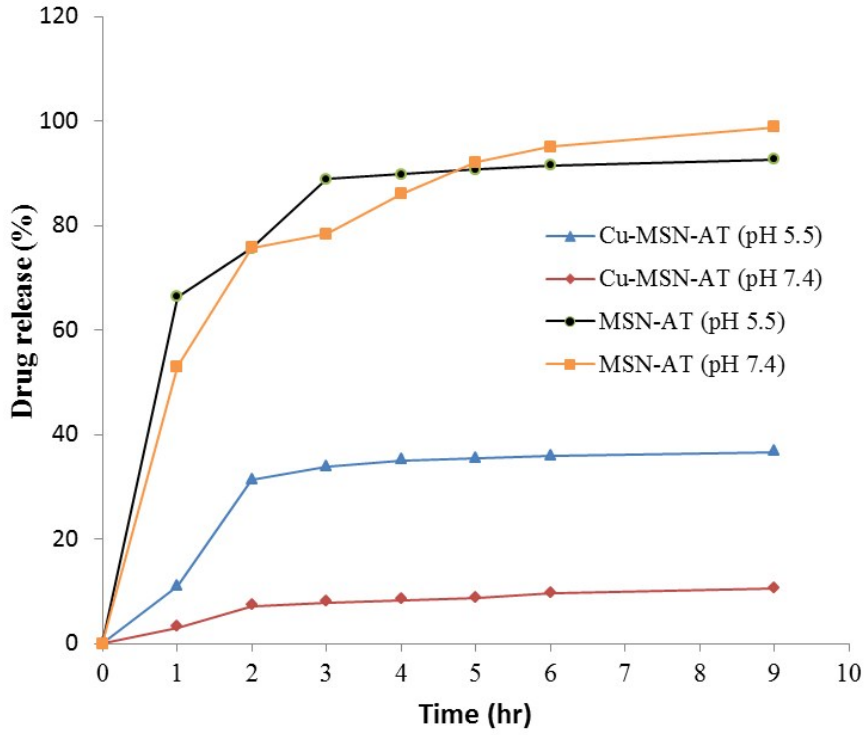

Fig. S5 Aminotriazole release from MSN and Cu-MSN at various time intervals in physiological simulated fluids (Phosphate-buffered saline (PBS) at a pH-5.5 and 7.4).

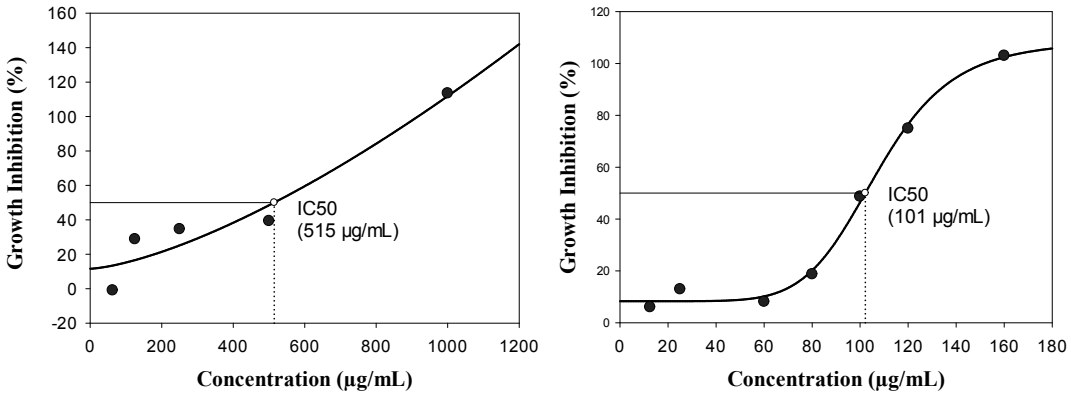

Fig. S6 IC_{50} from the SRB assay of (a) MSN and (b) MSN-AT complexes to HT-29 cells. Various concentrations of MSN nanocarriers were added to cells for 24 hours. Then, cells were fixed, stained with SRB, washed with dd-H_2O, subsequently solubilized

before reading the absorbance at a wavelength of 515 nm in Enspire multi-plate reader.

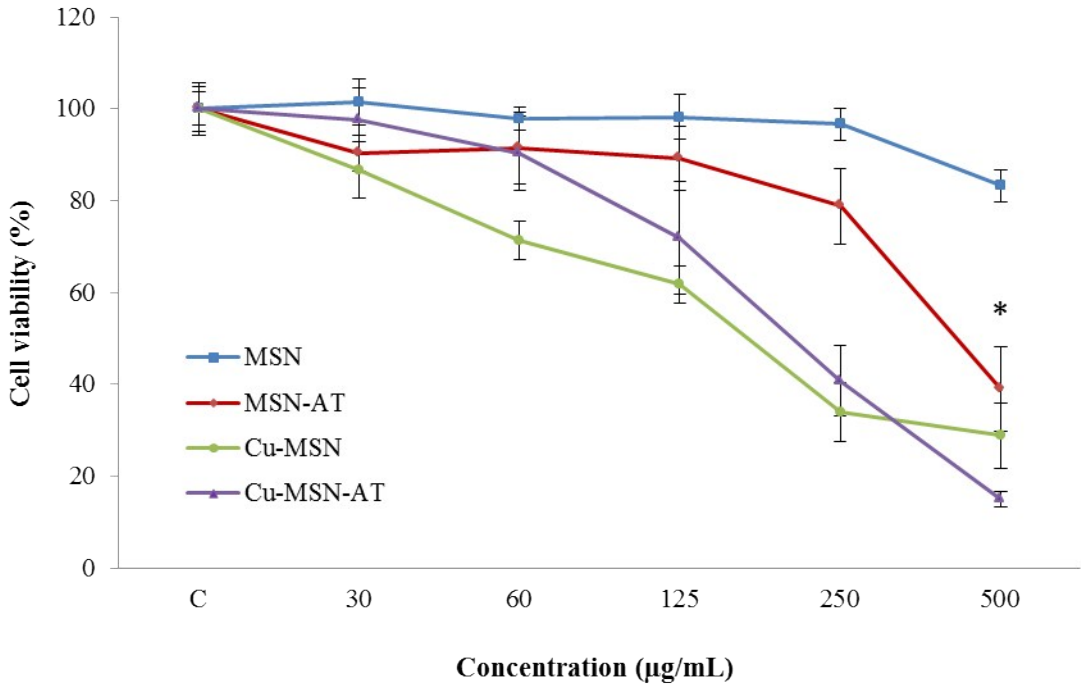

Fig. S7 Cell viability of normal fibroblast (3T3) cell line from the SRB assay of various nanomaterials. Cells were fixed, stained with SRB, washed with dd-H_2O, subsequently solubilized and finally absorbance was read at a wavelength of 515 nm. *represents $p<0.001$ (one way ANOVA using a Tukey test).

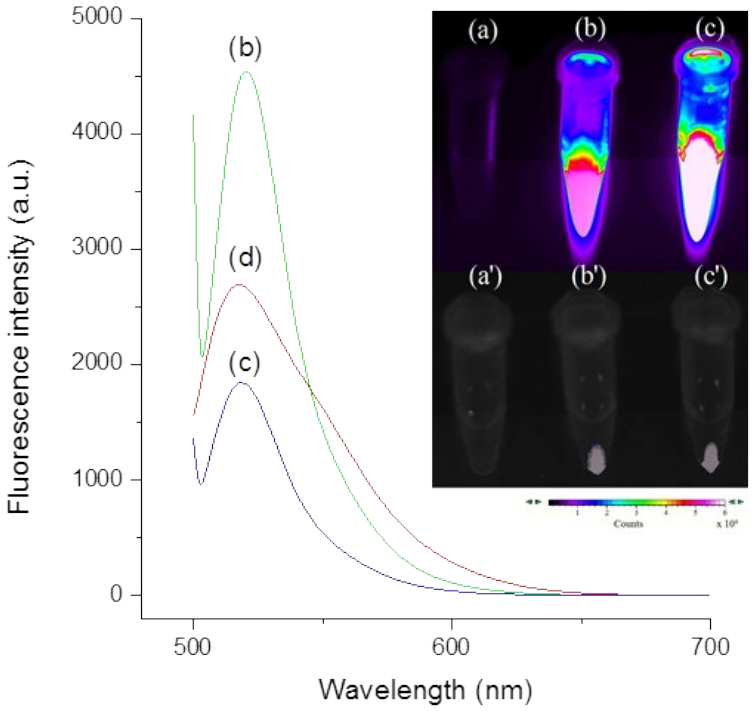

Fig. S8 Fluorescence spectrophotometric representation of (a) Cu-MSN, (b) FITC-Cu-MSN, (c) FITC-Cu-MSN-AT and (d) free FITC in ethanol. In the inset, sample images scanned using fluorescent imaging system in the top row (a, b, c) and the respective images of centrifuged nanoparticles in the bottom row (a‘, b‘, c‘) merged with the bright field view of the images.

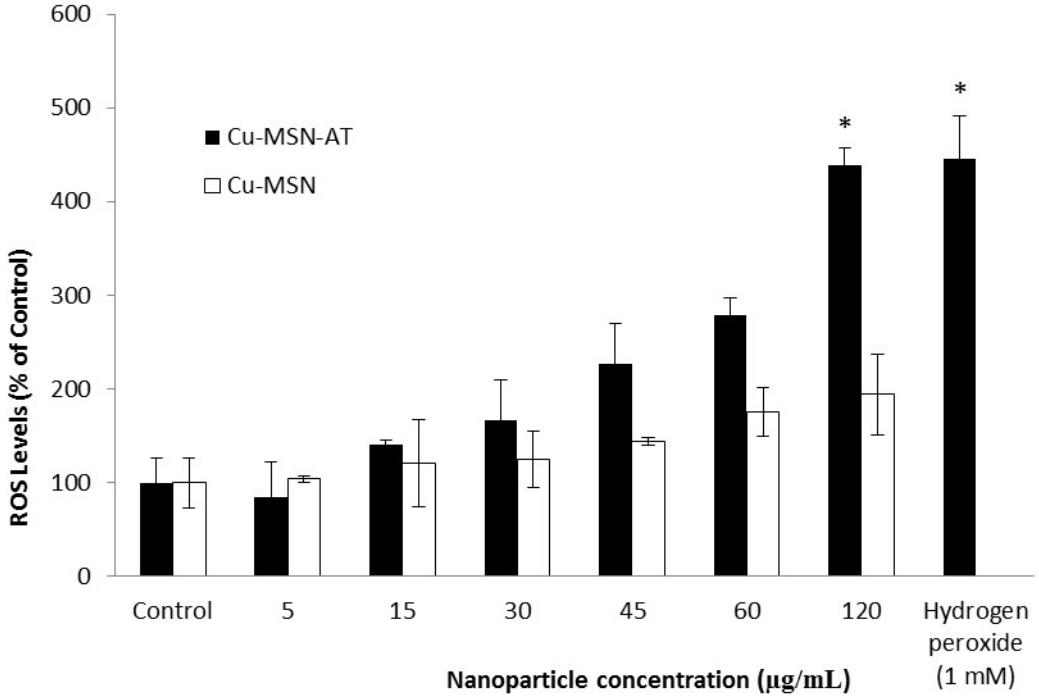

Fig. S9 Free radical determination measured by intensity of dichlorofluorescein (DCF) fluorescence. *represents $p<0.001$ (one way ANOVA using a Tukey test).

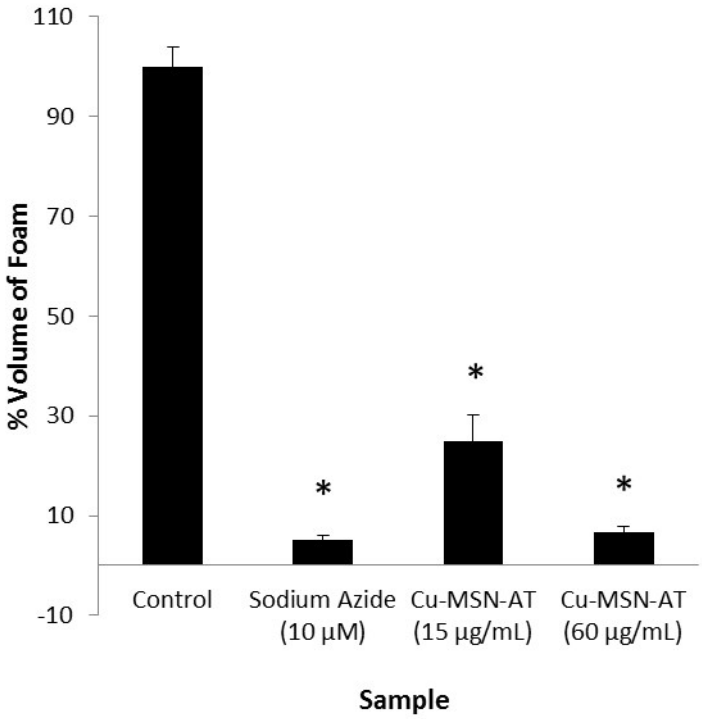

Fig. S10 Catalase activity by the foam test after the treatment by various concentrations of Cu-MSN-AT and the volume of foam generated in tube HT-29 cell line after adding 30% hydrogen peroxide (100 µL). *represents $p<0.001$ (one way ANOVA using a Tukey test).

EXPERIMENTAL METHODS

Preparation of FITC-Cu-MSN Sample

The FITC-conjugated APTS was synthesized by stirring FITC (2 mg) with APTS-EtOH solution (0.1 M in 10 mL of ethanol) for 24 hours in the dark. Separately, CTAB (0.58 g) was dissolved in NH_4OH (0.51 M, 300 mL) at 40 ^oC, and a dilute TEOS (0.2 M in 5.0 mL of ethanol) was added with stirring. The stirring was continued for 5 hours, then above prepared FITC-APTS solution (5.0 mL) and TEOS (1.0 M in 5.0 mL of ethanol) were added with vigorous stirring for another 2 hours. The solution was then aged at 40 ^oC for 24 hours. Nanoparticles were collected by centrifuging at 12,000 rpm for 18 minutes and then washed and re-dispersed with deionized water and ethanol several times. The surfactant templates were removed by extraction using ammonium nitrate in isopropanol (about 0.3 g of NH_4NO_3 in 50 mL of isopropanol at 85 ^oC for 24 hours).

Cell culture

To determine the anti-cancerous efficacy of 3-amino-1,2,4-triazole an irreversible catalase inhibitor, HT-29 (colon cancer) cell line and 3T3 (normal fibroblast) cell line was cultured in a RPMI-1640 medium and Dulbecco's Modified Eagle Medium (DMEM) respectively, supplemented with 10% (v/v) fetal bovine serum and penicillin

(100 units/mL)/streptomycin (100 µg/mL). Cultures were maintained throughout the process in a humidified incubator at 37 °C in 5% CO_2.

Cell viability assay (SRB assay)

Cellular viability was measured using SRB assay, which was reported previously.[1] Cells were inoculated into 96-well plates at a density of $1x10^4$ cells/well and incubated for cell attachment. After attachment one group of cells was fixed *in situ* with 25 µL of 50 % (w/v) trichloroacetic acid (TCA) to determine the cell number at the time the cells received the tested drug (T_0) and another group with medium alone, named as a control. The cells were treated with various concentrations (from 1-1000 µg/mL) of the samples of different modifications of MSN nanohybrids in FBS free medium and indicated as T_x, and incubated for 4 hours to allow for cellular uptake. Then, the equal volume of medium with 20% FBS was added as a supplement to avoid starvation of the cells and samples were incubated for another 20 hours. At the end of incubation, 50 µL of 50% (w/v) ice-cold trichloroacetic acid (TCA) was added to all T_X groups as well as the control and further incubated for 30 minutes at 4 °C. Finally, the medium was pipetted out and the cells were washed with dd-H_2O for four times and dried under air for 12 hours. Later, 100 µL of SRB (0.4% in 1 % acetic acid in water) was added into each well and incubated for 30 minutes, unbound SRB was washed with 1% acetic acid four times and SRB was

solubilized using a 10 mM Trizma base (pH-10.5) before the absorbance was eventually measured at 515 nm. The final results were expressed as a percentage growth inhibition and calculated using the following formula: $100-[(T_x - T_0)/(Ctl-T_0)]\times100$ (when $T_x \geq T_0$). GI_{50} (concentration of 50% cell growth inhibition) was defined as $(100 - [(T_x - T_0)/(Ctl-T_0)]\times100)= 50$ and calculated by the Sigma Plot software.

Cell uptake studies & quantification using fluorescent microscopy and flow cytometric analysis

Drug delivery efficacy of mesoporous silica nanoparticles was investigated by cell uptake study using FITC conjugated Cu-MSN-AT by visualization with fluorescent microscopy according to the reports available previously.[2] HT-29 cells were harvested at 80% confluence with a trypsin-EDTA complex solution and seeded into 6-well plates at a density $2x10^5$ cells/well. FITC-Cu-MSN and FITC-Cu-MSN-AT samples along with a control sample were incubated for 6 hours and then the cells were fixed with 3.7% paraformaldehyde and then, the culture was incubated eventually for 10 minutes with 0.5% triton-X 100 for cell wall dissociation; cell nuclei were stained with DAPI (100 µl, 0.1 mg/mL). Intermittently, cells were washed thrice with PBS for each step. Eventually the cell images were captured using fluorescent microscope.

Similarly, flow cytometric determination was performed as follows. The harvested cells were seeded at a density of $3x10^5$ cells/well. After 4 hours of incubation, the cells were treated with FITC-Cu-MSN (100 µg/mL) for a further 24 hours along with the control sample (i.e., media devoid of the nanoparticles). Cells were collected in a tube and centrifuged at 400xg for 5 minutes, washed once with PBS, re-suspended and fluorescence was quantified using flow cytometry. The samples were immediately analyzed after treatment by measuring fluorescence using Accuri BD biosciences flow cytometry with a laser set at 530 nm in a 3 decade pulse area. The forward and side light scatter profiles were adjusted and fluorescence parameters were calculated by collecting logarithmic amplification data.

Free radical determination

In vitro free radical quenching of Cu-MSN-AT nanohybrids was determined precisely measuring the fluorescent product (oxidized form, i.e., DCF) of DCF-DA in the presence of free radicals generated in the cells, as reported previously[3, 4] with slight modification. Prior to the free radical determination, DCF-DA was activated using an ethanolic stock solution and added in the wells and incubated for 30 minutes in the dark. The reaction mixtures containing the nanohybrid in various concentrations were placed in 25 mM sodium phosphate buffer (pH-7.2) and incubated for 4 hours. The resultant absorbance of the whole reaction mixture (activated DCF-DA along

with the nanohybrids), in addition to the positive control (hydrogen peroxide (1mM)) was measured using an Elisa reader at E_x/E_m 485/528 nm. Flow cytometric measurements were performed by seeding the cells at a density of $3x10^5$ cells/well, and after 24 hours of incubation the cells were treated with respective concentrations of Cu-MSN_AT for a further 24 hours along with the control i.e., media devoid of the nanoparticle.

Comet assay

DNA fragmentation was determined by using the most reliable technique, alkaline comet single cell gel electrophoresis, according to the manufacturer's instructions and the technique was further optimized with the help of a previous report.[4, 5] HT-29 cell lines were treated with the Cu-MSN-AT sample. Cells were harvested and pooled in 1% low melting point agarose at a ratio of 1:10 (v/v) at 37 ^oC and immediately layered on custom frosted slides which feature a clear centered window and the rest of the procedure was performed following manufacturer's instructions. The gel was run in the alkaline electrophoresis buffer (0.2 N NaOH, 0.5 mM EDTA, pH-12.5) for 20 minutes at 21 Volts and 350 mA. The slides were then washed in water and ethanol for 5 minutes to reanneal the DNA and finally the dried smears were stained with SYBR green (1 mg/mL) for 30 minutes and micrographs were captured using a

fluorescence image analysis equipped Olympus microscope with Nikon CCD camera (Japan).

Lipid peroxidation

Lipid peroxidation of cell membrane lipids is an indicator of an oxidative stress. The generated thiobarbituric acid reactive substances (TBARS) such as lipid hydro peroxides and aldehydes (ex. Malondialdehyde (MDA)) in the cell culture media and cell lysate combine with thiobarbituric acid (TBA) in a 1:2 ratio to form a fluorescent adduct. TBARS were expressed as MDA equivalents by following a previously reported procedure with slight modification.[4, 6] Cells were treated with various concentrations of Cu-MSN and Cu-MSN-AT (15, 30, 45 and 60 µg/mL) and incubated for an hour along with 10 mM of diethyl maleate (DEM) used as a positive control. HT-29 colon cells from prepared flasks were harvested after attaining 80% confluence and seeded at a density of 5 x 10^5 cells/mL in RPMI-1640 cell culture media (2 mM L-glutamine, 10% FBS). After 24 hours of incubation, the cell culture media was replaced with respective test nanomaterials suspended in PBS along with negative and positive controls and incubated at 37 ^oC in 5% CO_2. After 3 hours of incubation, the supernatant (200 µL) was taken and TBARS assaying was performed *in vitro*. To the supernatant, mixture containing 400 µL of 0.67% TBA/0.01% BHT in 2.5% TCA and 200 µL of 15% TCA was added. The mixture was heated at 95 ^oC for 30

minutes, allowed to cool down and then a complex of MDA-TBA was extracted using n-Butanol (500 µL). Fluorescence was measured eventually at (Ex/Em = 532/553 nm) along with the reference recorded to a reagent blank.

Intracellular hydrogen peroxide levels

Hydrogen peroxide levels in cells can be estimated using the following method as reported previously with slight modification.[7] In catalase activity assays, the cell pellets were thawed rapidly and then sonicated three times at 3 minutes per cycle in a Bransonic sonicator. Spectrophotometric measurement of catalase activities was carried out using different concentrations of aminotriazole loaded Cu-MSN. The assay was initiated by adding 500 µL of 30 mM H_2O_2 stock solution in PBS pH-7.0, and the loss of the absorbance at 240 nm at 25 ^oC, which can be correlated to the catalase activity was monitored by a UV spectrophotometer,

Catalase activity (foam test)

Catalase activity was measured by establishing the correlation with the generated foam as reported previously, with slight modifications.[8] HT-29 cancer cells at a density of 5 $x10^6$ cells/well were re-suspended in 100 µL of phosphate buffered saline (PBS) and placed in a 15 mL falcon tube with graduations (17 x 120 mm, BD Falcon; Franklin Lakes, U.S.A). After 4 hours of post treatment with Cu-MSN-AT, 100 µL of 1% triton X-100 and 100 µL of hydrogen peroxide (30%), respectively, were

added to the solutions and then incubated at room temperature. Subsequently the height of O_2-forming foam that remained constant for 15 minutes in the tube was the volume of foam finally noted after completion of the reaction.

Mitochondria membrane potential (MMP)

The MMP was ratio-metrically determined by the indicator JC-1 stain (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide) and observed in the treated cells by correlating the changes in the red/green shift of coloration. Cultured HT-29 cell line at a density $1x10^4$ cells/well in a 96-well plate was subjected to the Cu-MSN-AT treatment and after 24 hours of post treatment, the indicator JC-1 stain was added at 5 µM and incubated for 30 minutes at 37 ^oC. Finally, the dye was cleared off and washed twice with PBS; subsequently, cells were observed under the fluorescent microscope for dye aggregates, which represents the mitochondrial potential.

Western Blot

Apoptosis of mesoporous silica nanoparticles was investigated by performing western blottting using aminotriazole conjugated Cu-MSN via visualization using western blot chemiluminescence reagent. HT-29 cells were harvested at 80% confluence with trypsin-EDTA solution and seeded into a plate (6-well) at a density $5x10^5$ cells/well and incubated for 24 hours and following successive attachment,

cells were treated with various concentrations of mesoporous silica nanocontainers loaded with aminotriazole and incubated for a further 24 hours. The cells were collected and homogenized in a GE Healthcare Mammalian Protein Extraction Buffer. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted onto PVDF membrane (Immuno blot PVDF membrane, Bio-Rad U.S.A). The blots were blocked with 5% non-fat milk in TBST saline (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, and 0.05 % Tween-20) at RT (room temperature) for an hour and incubated with the appropriate primary antibody at 4 °C overnight. After a wash, the blots were re-incubated with peroxidase-conjugated secondary antibody for one hour following TBST washes. Bands were monitored using a western blot chemiluminescence reagent (Western ECL Substrate, Bio-Rad, U.S.A).

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