Supporting Information

Silver Nitroprusside Nanoparticles for Breast Cancer Therapy: *In vitro* and *In vivo* Approach

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Materials and Methods

Sodium nitroprusside (SNP) (Na_2Fe $(CN)_5NO$), silver nitrate ($AgNO_3$), MTT:3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide reagent, dihydroethidium (DHE),5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (DCFDA), methyl-β cyclodextrin, sodium azide (NaN_3), wortmannin, doxorubicin (Dox), ribonuclease (RNase), RIPA buffer, propidium iodide (PI), phosphate buffer saline (PBS), sodium dodecyl sulphate (SDS), streptomycin, bovine serum albumin (BSA) were brought from Sigma Aldrich Chemicals. DMEM: Dulbecco's modified Eagle's Medium, FBS: Fetal bovine serum, RPMI: Roswell Park Memorial Institute media, acrylamide, bis-acrylamide were brought from HiMedia. Monensin and amiloride were procured from Alfa Aesar. The PVDF membrane was brought from Merck Milipore. Bradford reagent and triton X-100 was obtained from Genetix Biotech Asia Pvt. Ltd. Dimethyl sulphoxide (DMSO) was obtained from Rankem (India).

Primary antibodies **(Cell Signaling Technology):** β-actin (13E5) rabbit Ab (cat. no. #8584), Bax rabbit Ab (#2772), cleaved caspase-9 (D8I9E) Rabbit Ab (#20750), caspase-9 (C9) Mouse Ab (#9508), caspase-3 Rabbit Ab (#9662), cleaved caspase-3 (D3E9) Rabbit mAb (#74860), STAT3 (124H6) Mouse mAb (#83541), NF-κB (D14E12) Rabbit mAb (#8242), cytochrome c (D18C7) Rabbit mAb (#11940),CD31/PECAM-1 (89C2) Mouse mAb #3528, anti-Ki67 (9129T), Ki-67 (D3B5) Rabbit mAb (#9129).

Secondary antibodies: Anti-rabbit (HRP) IgG (31460) and anti-mouse (HRP) IgG (31430) were obtained from the Cell Signaling Technologies. Chemiluminescent substrate and protein ladder were brought from Thermo Fischer Scientific.

Kits: FITC Annexin V Apoptosis Detection kit has obtained from Cayman. Mitochondria membrane potential assay kit, TUNEL and TNF- α assay kit has been brought from Sigma Aldrich Chemicals, USA. The ELISA kits (IL-4, IL-6and INF- ϒ) have been procured from PUREGENE.

CHARACTERIZATION TECHNIQUES

XRD (X-ray Diffraction Analysis)

The XRD is a characterization technique to determine the crystallographic nature and phase purity of the material. It detects changes in the diffraction peaks and analyzes the crystal structure. The crystalline nature of AgNNPs was analyzed by XRD analysis through the use of Bruker AXS D8 Advance Powder X-ray Diffractometer (byCuKαλ=1.5406 A radiation) as per previous reports.[1-3] The data was obtained in the 2θ range (10° to 80°) in the continuous scanning mode.

DLS (Dynamic Light Scattering)

The hydrodynamic diameter (size)and surface charge(zeta potential) of AgNNPs was performed by DLS instrument [(Anton Par)Litesizer500 particle analyzer] according to published literature.[2, 4] Briefly, AgNNPs stock solution prepared in tris-EDTA (1mg/mL) and diluted with miliQ water (1:9 v/v).

FTIR (Fourier Transformed Infrared Spectroscopy)

FTIR analysis of sodium nitroprusside, silver nitrate and AgNNPs was performed using Thermo Nicolet Nexus 670 spectrometer to determine the changes in the stretching frequenciesofthe functional groups. The FTIR spectra were performed in diffuse reflectance mode in KBr pellets at a range of 400-4000 cm^{-1}.[1, 5]

SEM (Scanning electron microscopy) and FE-SEM (Field Emission- Scanning Electron Microscopy)

The SEM facilitates to understand the microstructural surface composition and morphology of nanoparticles. Scanning electron microscopy (SEM) of powdered AgNNPs was performed using SEM Hitachi S-3000N, Japan. [4]

The detailed high resolution surface morphology of AgNNPs was performed using field FE-SEM analysis by FESEM, JEOL 7610F.

TGA (Thermogravimetric Analysis) and DTG (Derivative of Thermogravimetric analysis)

The TGA/DTG was carried out to analyze presence of water molecules and thermal stability of AgNNPs(TGA/SDTA851c, from METTLER TOLEDO, Switzerland) from 25 to 1000°C at 10 °C/min in nitrogen atmosphere. [1, 2]

TEM(Transmission electron microscopy)

The size, shape and morphology of lyophilized AgNNPs were evaluated by TEM (with the help of Tecnai G2 F30 S-Twin Microscope operated at 100 kV).[1, 3] Initially, AgNNPs were dissolved in ethanol, coated over copper grid, allowed to dry and submitted for TEM analysis.

EDAX (Energy-dispersive X-ray) analysis was carried outby FEI Quanta 200 ESEM joined with an Oxford X-MaxN 20 EDXS detector at an accelerating voltage of 25 kV.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES)

The ICPOES experiment was performed to determine the silver ion content in several *in vitro* (cellular uptake pathway) and in *vivo* experiments (biodistribution) as per earlier reports.[1, 3]

In vitro study

Cell viability assay using MTT reagent

Briefly, the cancer cells (MDA-MB-231,4T1, B16F10, PANC1,) and normal cells (HEK-293, EA.hy926) were grown in a 96 well tissue culture plates (approximately 8000cells/well) for 24 htill80-90% confluency. The cells were then treated with (1-20 µg/mL) AgNNPs for 24 h. Then, the media was replaced by (0.05 mg/mL) MTT reagent in each well. The plates were then keptfor another 4 h at 37°C in dark condition. Later, the MTT solution was discarded and mixture of DMSO and methanol in 1:1 (v/v)ratio was added in each well to solubilize the formazan crystals. The absorbance intensity of the solution was determinedusing Synergy H1 multimode plate reader system at 570 nm. Finally, the percentage cell viability was expressed asnormalized values with respect to untreated cells.

Inflammatory cytokine detection by ELISA

Briefly, the AgNNPs (1 mg/kg b.w./day) was intraperitoneallyadministered into the mice (n=3). After 24 h, blood was collected from the retro-orbital plexus and serum was isolated from the blood through centrifugation at 4000 rpm, 20 mins, 4°C. The serum wasthen used to detect the expression of inflammatory cytokines such as IL-6, IL-4, IFN-γ and TNF-α using ELISA kit. The individual ELISA assays were performed as per the manufacturer's protocol. The absorbance of the samples was analyzed at 450 nm with the help of Synergy H1 multimode plate reader. The serum cytokine concentration (pg/mL) was determined from a standard curve plotted using cytokine standards.

Result and discussion

Fourier Transformed Infrared Spectroscopy

The IR stretchingfrequencies (Figure S2) obtained from SNP are spectrum a: 3959.50, 3871.79, 3617.39, 3205.01, 2923.62, 2824.67, 2597.93, 2548.05, 2408.73, 2173.97, 2143.78, 2110.20, 1935.86, 1616.55, 1384.28, 1322.67, 1159.71, 983.98, 865.73, 663.27 and 454.76. The IR stretchingfrequencies obtained from $AgNO_3$ are spectrum b: 3451.45, 2922.67, 2426.13, 1762.46, 1622.81, 1383.95 and 824.12. The stretching frequencies of AgNNPs are spectrum c: 3845.83, 3455.65, 2175.72, 2141.14, 1935.12, 1619.45, 1019.12 and 660.67. Since there are narrow absorption band in the spectrum range of υ(OH) vibrations (observed at 3845.83), it is likely that the AgNNPs are anhydrous in nature as evidenced by the TGA spectra analysis **(Fig. S1d)** where there is no weight loss observed below 200°C. The υ(NO) vibrations are obtained at 1935.12 cm^{-1}. While, the υ(CN)vibrations occurred at 2175.72 and 2141.14 cm^{-1} which are ascribed as equatorial and axial CN functional groups, respectively.[6] The low stretching frequency of the axial CN group indicates that it either unlinked to silver atom or the bonds between them are relatively weak as compared to the equatorial CN group. The FTIR spectrum revealed that the two main peaks of nitroprusside which are 2173 and 1935 cm^{-1} assigned to CN and N=O functional group shifted to 2175 and 1935 in AgNNPs, respectively. Altogether the results displays that the silver ions almost replace the sodium ions in AgNNPs. All peaks are almost in corroboration with earlier literature. [2, 6]

Table S1: The percentage value of elements present in AgNNPs. The raw data obtained from EDX analysis.

Element	Series	Net	[wt.%]	[norm. wt.%]	[norm. at.%]	Error in wt.% (3 Sigma)
Silver	L-series	10691	2.756361	2.756361	0.320507	0.907911
Iron	K-series	3569	0.719625	0.719625	0.161622	0.149166
Carbon	K-series	10292	91.34979	91.34979	95.39417	8.825944
Nitrogen	K-series	2854	0.603931	0.603931	0.54081	0.139066
Oxygen	K-series	844	4.570295	4.570295	3.582894	0.701763
		Sum:	100	100	100	

Table S2: Different pathway inhibitor for cellular uptake pathway of AgNNPs in 4T1 cells.

Pathway	Inhibitor	Treatment	Incubation period (min)
Phagocytosis	Wortmannin	50 nM	45
Energy dependent	Sodium azide	0.1%	60
Macropinocytosis	Amiloride	50µM	30
Clathrin and caveloe independent	Monensin	30µg/mL	30
Caveloe dependent	Methyl-β cyclodextrin	5mM	15
Clathrin dependent	Chloropromazine hydrochloride	10µg/mL	15 min

Figure S1

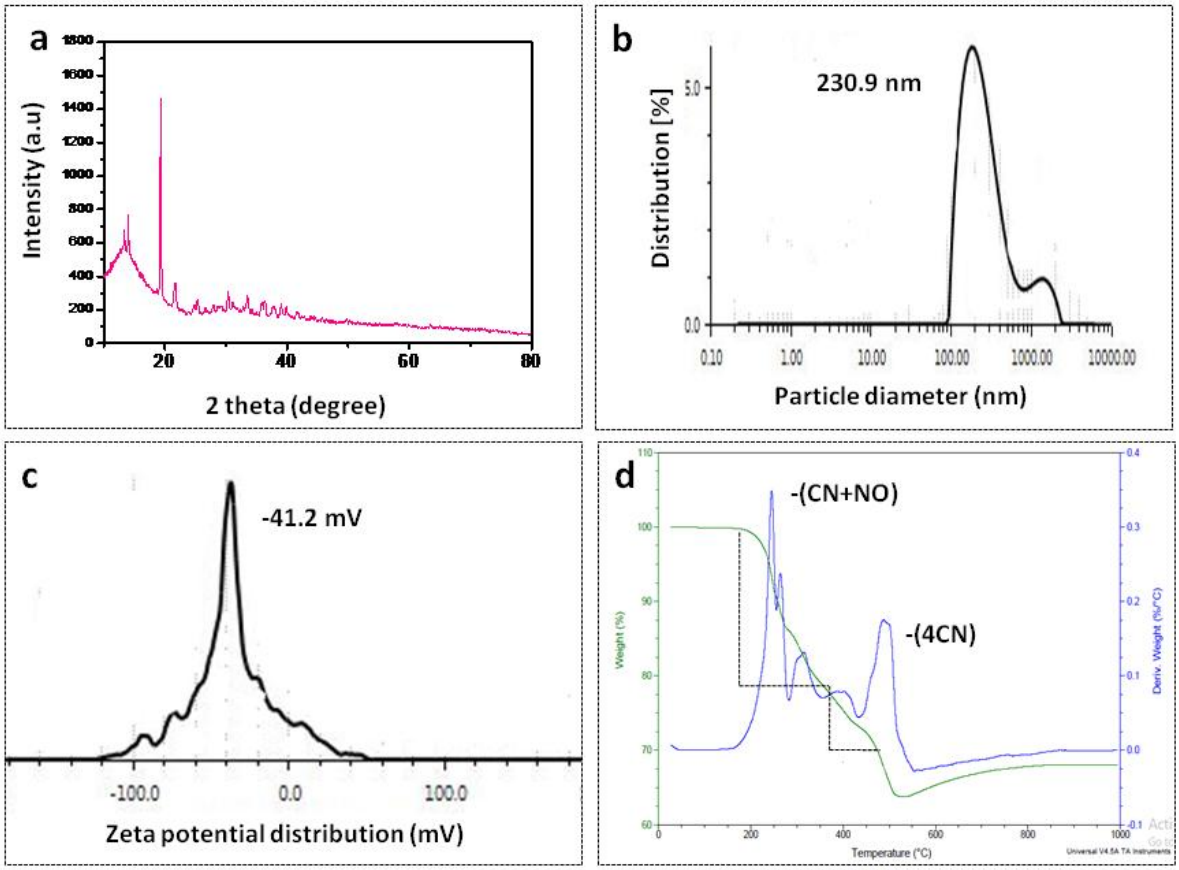


Fig.S1: a) XRD spectrum of AgNNPs synthesized by co-precipitation method depicting crystalline nature.The DLS of AgNNPs shows b) hydrodynamic size is around 230 nm and b) zeta potential nearly -41.2 mV. d)Thermogravimetric (green) and derivative of thermogravimetric (blue) spectra depicts the thermal behavior of functional groups present in AgNNPs. The spectra reveal the stepwise decomposition of AgNNPs occurred in between 200-550℃.

Figure S2

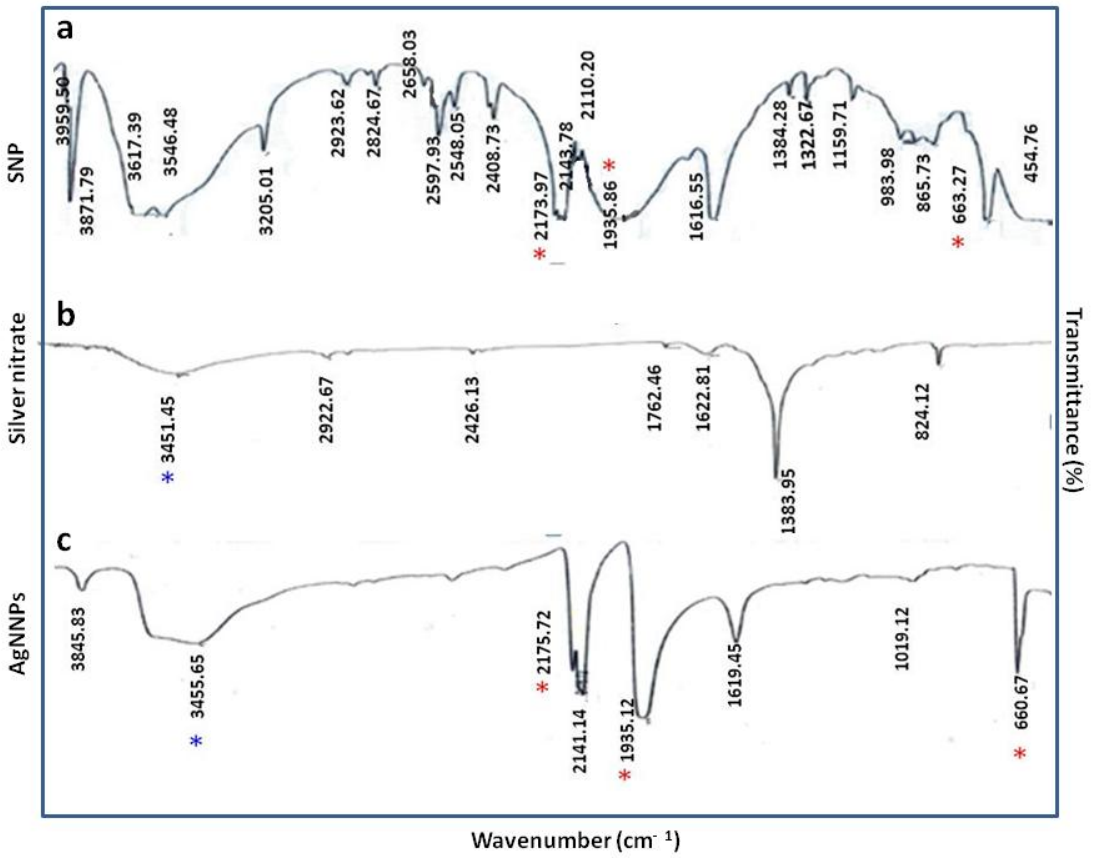


Fig. S2: FTIR spectra of AgNNPs indicating involvement of several functional groups in the formation of metal nitroprusside nanoparticles. Spectrum a: SNP, b: silver nitrate, c: AgNNPs. The two main peaks of AgNNPs 2173.97 and 1935.86 cm^{-1} shifted to 2175.72 and 1935.12 respectively which are assigned to CN and N=O functional group.

Figure S3

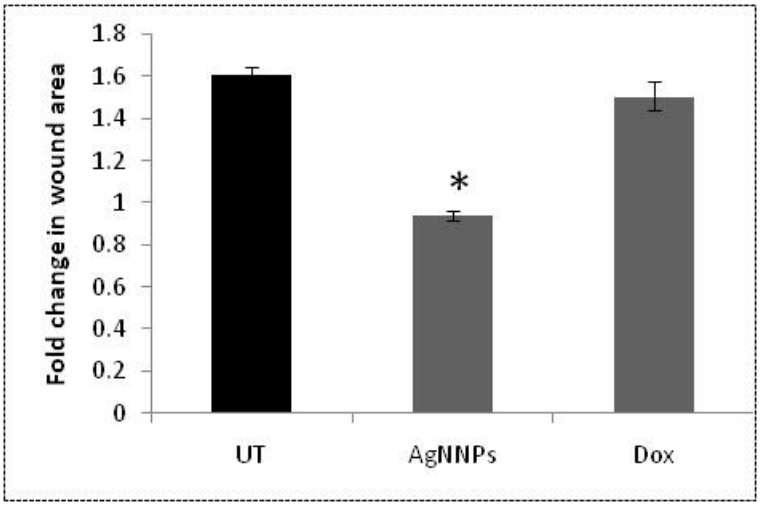


Fig. S3: Histogram represents the fold changes in scratch wound area after AgNNPs treatment quantified using IMAGE J software. This study was performed thrice and represented in mean ± SD. Significant differences from untreated (UT) cells are observed (*$p < 0.05$).

Figure S4

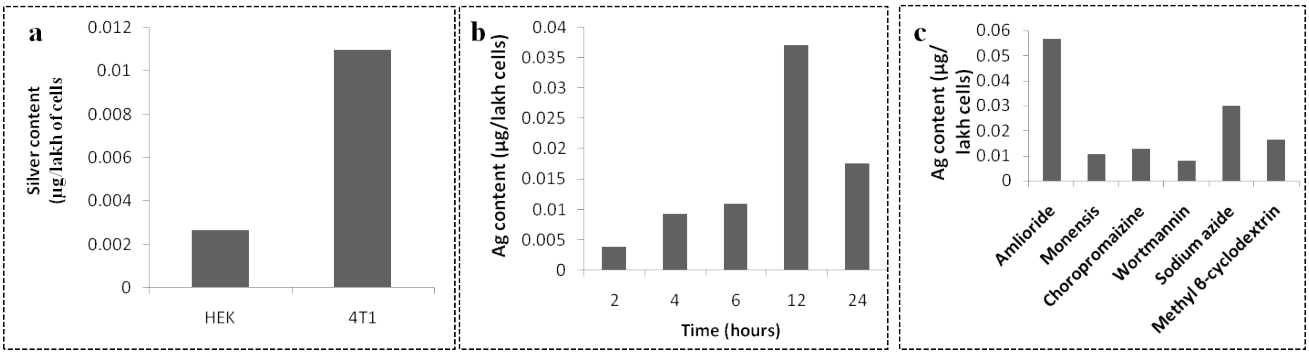


Fig. S4: a) The AgNNPs uptake variation in terms of silver content compared in the cancer cell (4T1) and normal cell (HEK-293) for 6 h determined by ICPOES analysis.It demonstrates more uptake occurs of AgNNPsin 4T1 cells as compared to the HEK-293b) The uptake study of AgNNPs in 4T1 cells in a time-dependent manner (2,4,6,12 and 24 h) determined by ICP-OES analysis. c) The determination of cellular uptake pathway of AgNNPs in 4T1 cells. It is carried out by incubation of cells with different inhibitors (amlioride, monensin, chloropromaizine, Wortmannin, sodium azide, methyl β-cyclodextrin) for respective time period followed by incubation with AgNNPs. The data is represented interms of Ag content (µg/lakh cells. These are all pulled data hence, statistical significance is not provided.

Figure S5

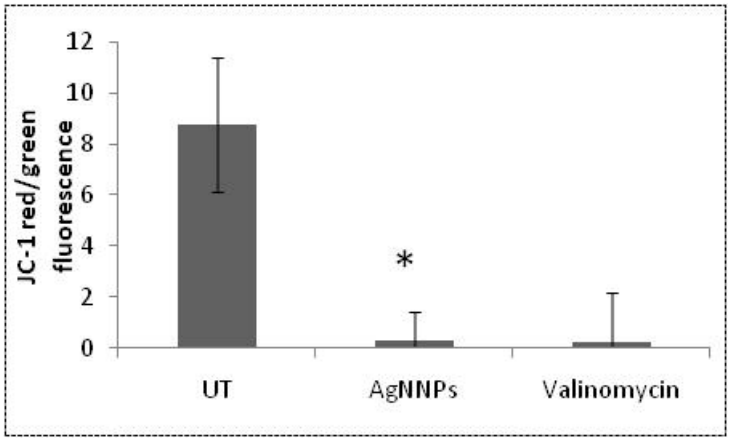


Fig. S5: Histogram represents the quantitative ratio of red to green fluorescence of JC-1 signal in MDA-MB-231 cells after AgNNPs treatment. The experiment is performed thrice and expressed as mean±SD.Significant differences from untreated (UT) cells are observed (*$p < 0.05$).

Figure S6

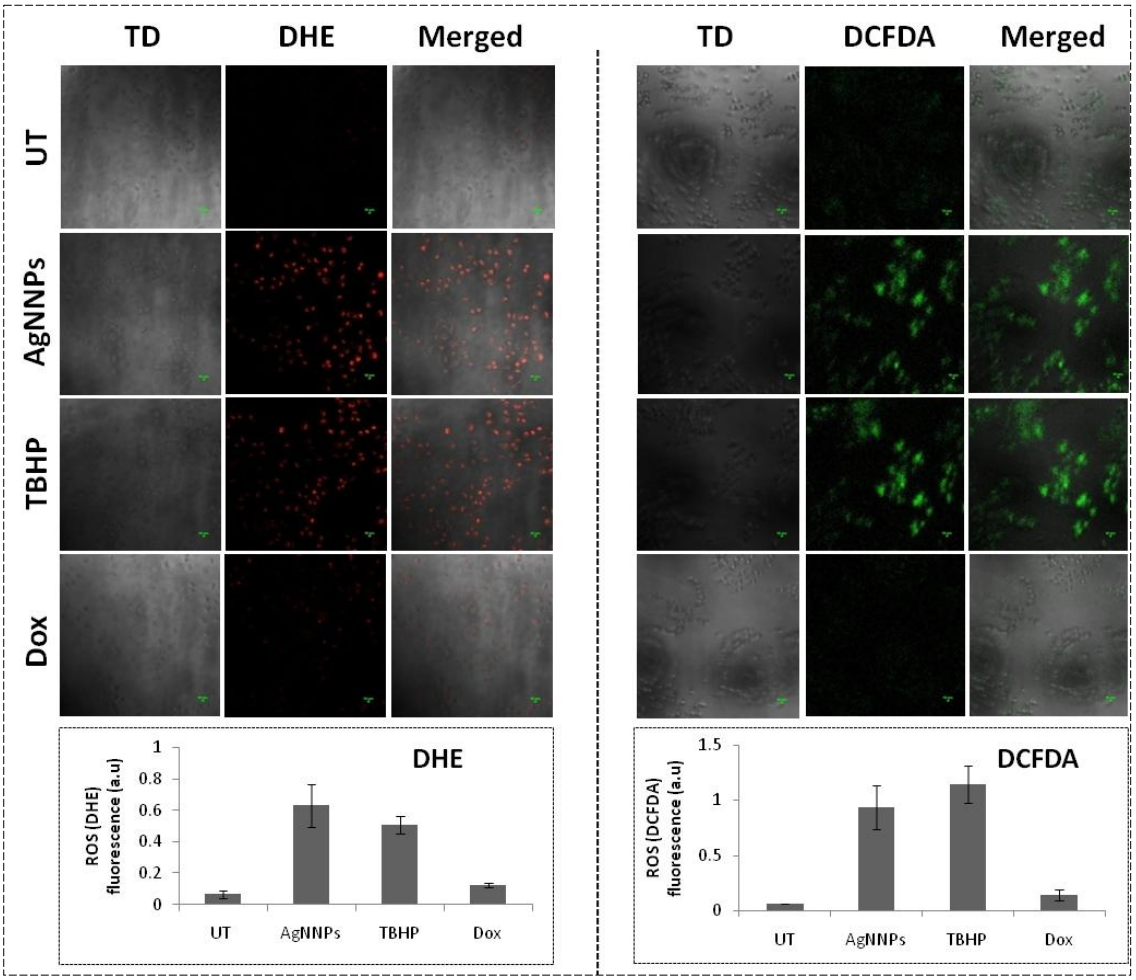


Fig. S6: Determination of the intracellular (H_2O_2 and $O_2^{\cdot-}$) ROS generation in MDAMB-231 cells using DCFDA and DHE reagent, respectively. For **panel** I: DHE; II: DCFDA; **Row**: I: UT; II: AgNNPs(4µg/mL); III: TBHP; IV: Dox (1µM). Higher intensity of green and red fluorescence shows the more formation of hydrogen peroxide and superoxide anion respectively in MDA-MB-231 cells by treatment of AgNNPs. The above images were taken at 20X magnifications by Confocal microscopy (laser 405nm, 561nm) scale bar=10µm. The histogram represents quantification of ROS (H_2O_2 and $O_2^{\cdot-}$). The ROS experiment was carried out thrice and represented in mean ± SD.

Figure S7

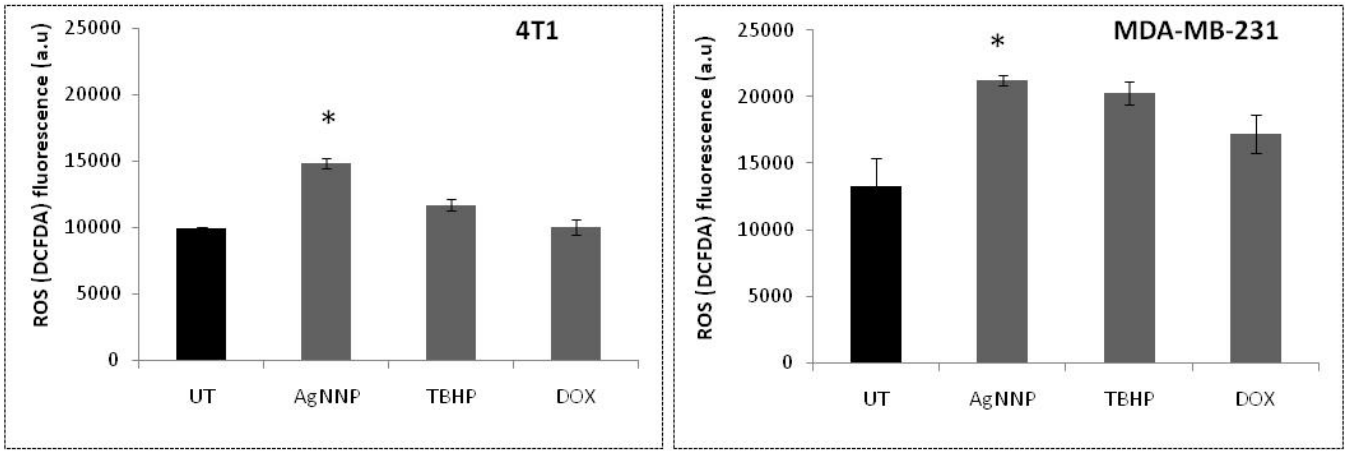


Fig. S7: Determination of the intracellular ROS (H_2O_2) production in (a) 4T1 and (b) MDA-MB-231 cells using DCFDA reagent after treatment with AgNNPs. The TBHP (10µM) and Dox is used as a positive control. The AgNNPs exhibits more ROS generation as compared to untreated. The experiment was performed thrice and represented in mean ± SD. The significant variations were observed from untreated cells (*$p<0.05$).

Figure S8

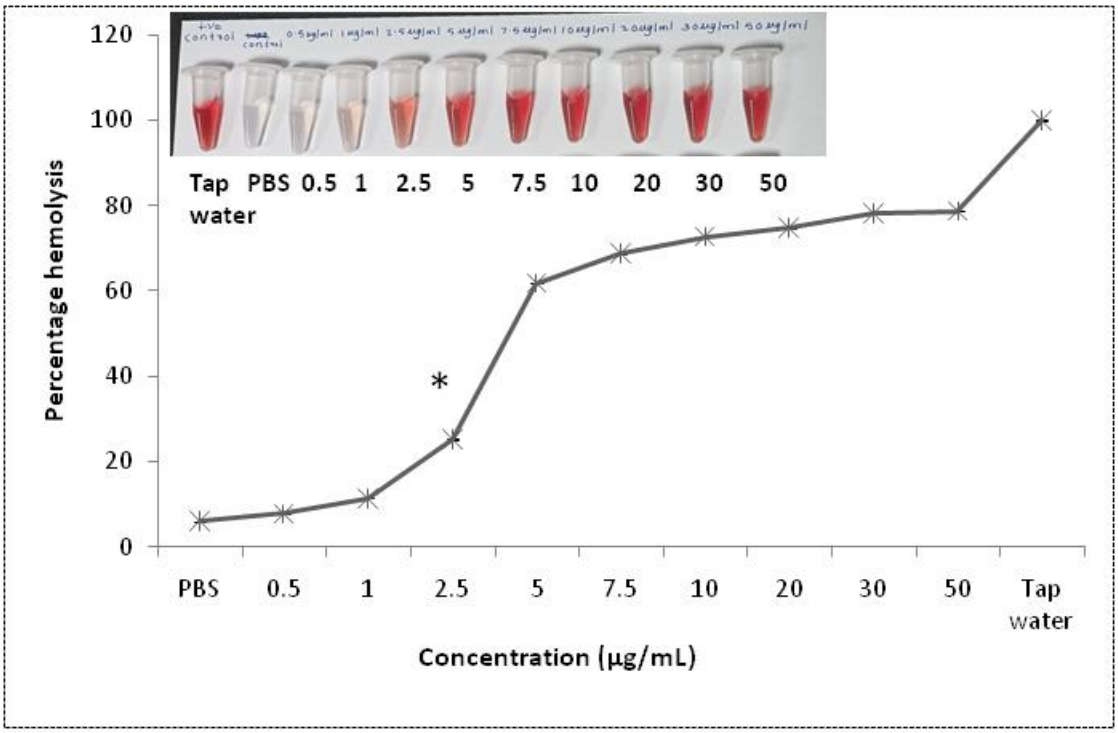
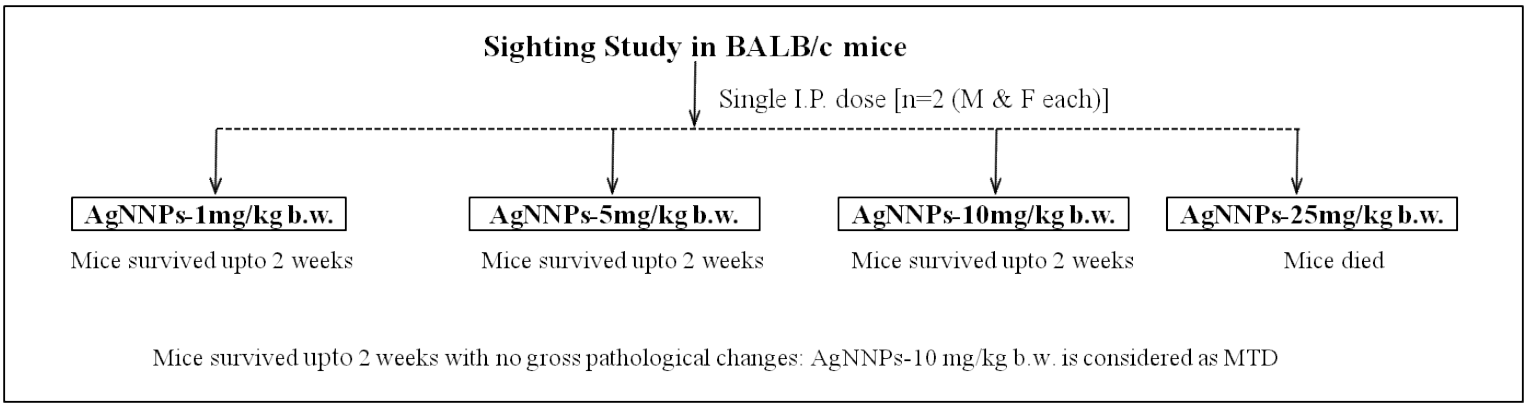


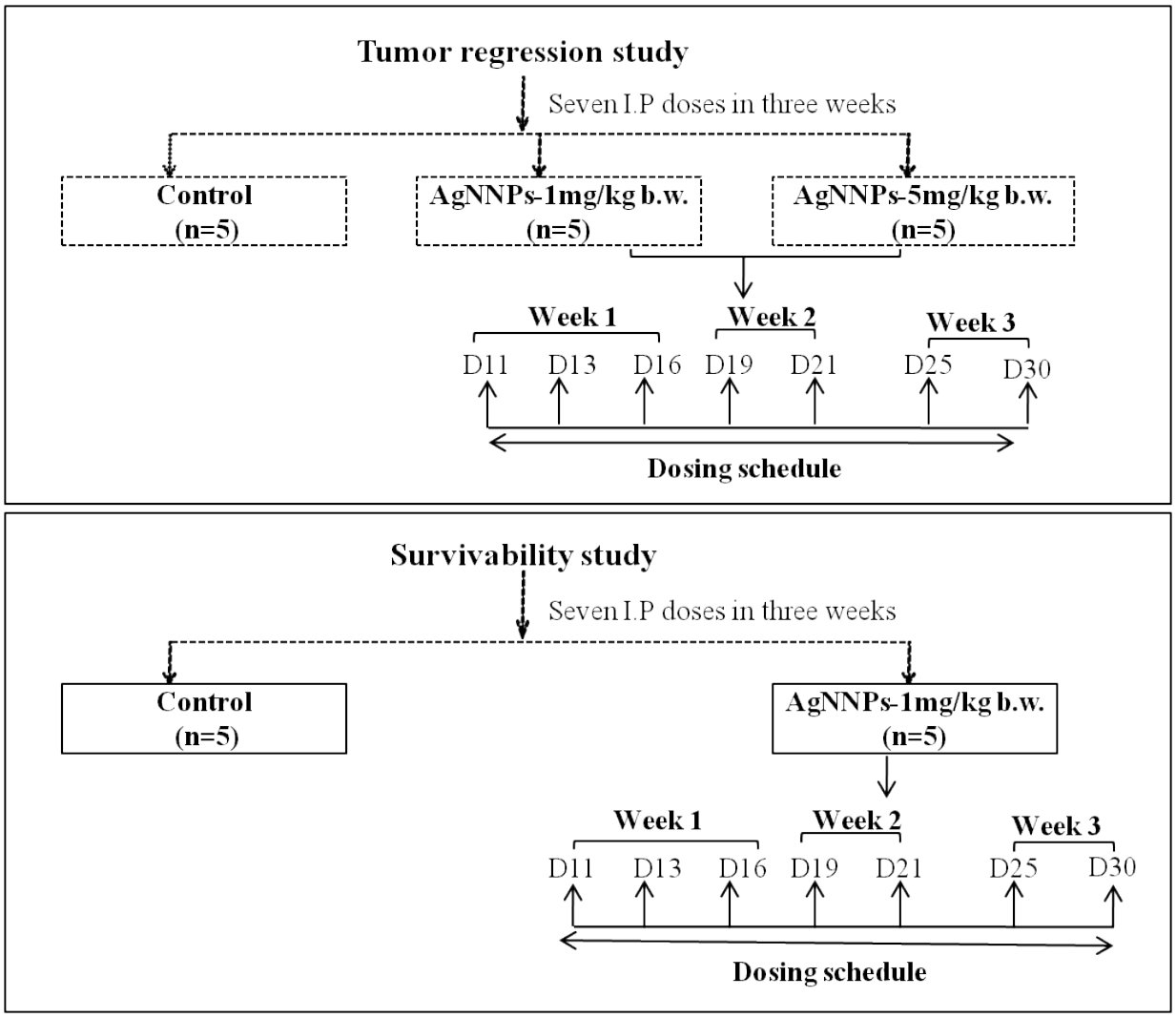
Fig. S8: The hemolysis assay using mice blood indicating the hemocompatible nature of AgNNPs (0.5-50 µg/mL). The inset optical picture demonstrates positive control has more hemolysis as compared to other treatments of AgNNPs. The following experiment was performed thrice and represented in mean±SD. A significant difference was observed from the negative control (*$p<0.05$).

Scheme 1



Scheme S1: Scheme for sighting study of AgNNPs performed in BALB/c mice in a dose dependent manner from 1-25mg/kg b.w. to determine the (MTD) maximum tolerable dose.

Scheme 2



Scheme S2: Schematic diagram of tumor regression and survivability study of AgNNPs in BALB/c female mice.

Figure S9

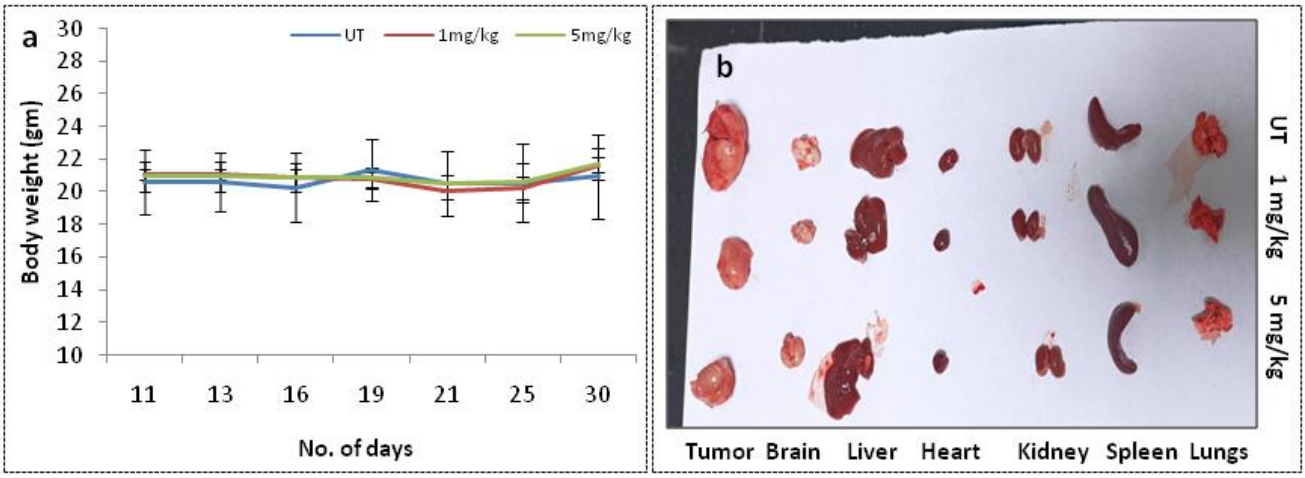


Fig. S9: a)The average body weight (gm) of all mice. It demonstrates no significant changes in body weight of all mice. b) Optical pictures of the organs (brain, liver, heart, kidney, spleen, andlung) and tumor tissue after sacrificing the mice.

Figure S10

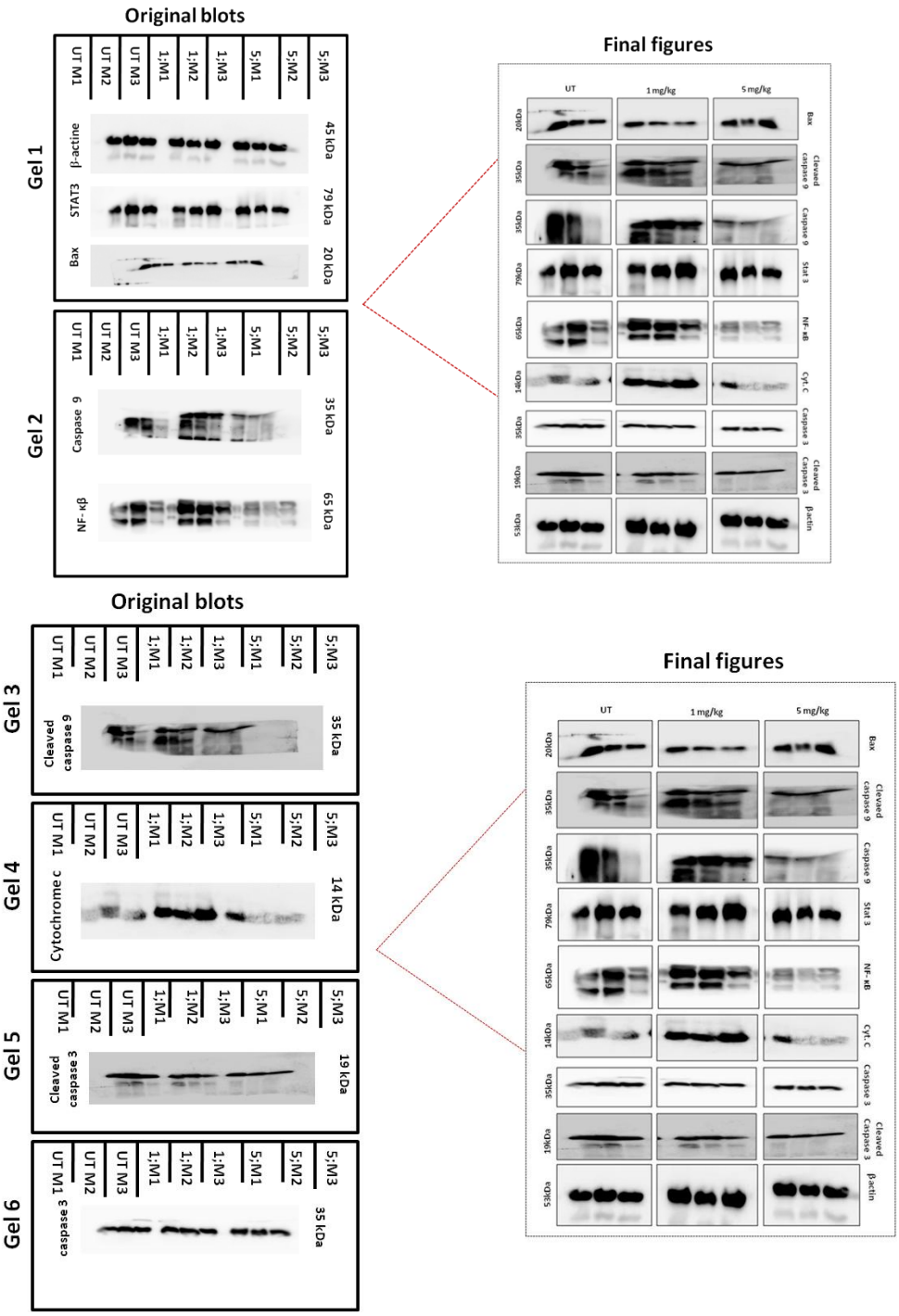


Fig.S10: Original western blots of tumor tissue (UT, 1mg/kg, 5mg/kg) along with cropped images of proteins (Bax,cleaved caspase 9, caspase 9 , STAT 3, NF- κB, cytochrome c, caspase 3 and cleaved caspase 3). Same source of tissue lysate was used for all protein expressions in the western blot experiments.

Figure S11

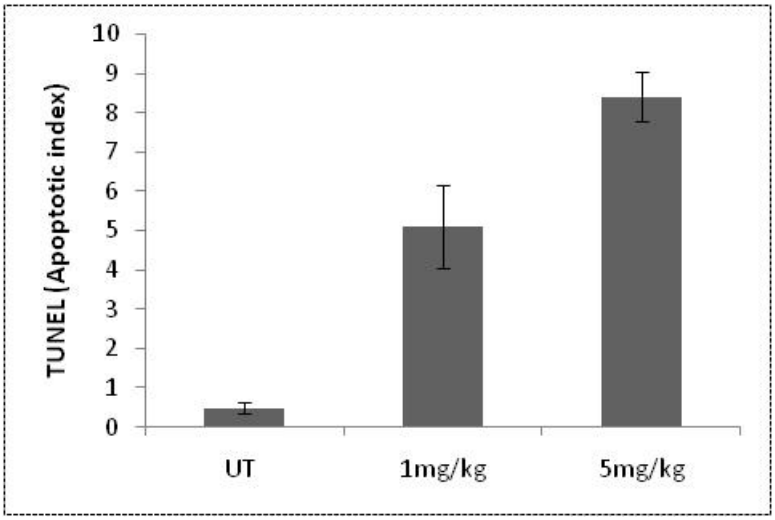


Fig. S11: Histogram represents the apoptotic index of TUNEL assay of tumor samples using AgNNPs and represented in mean ± SD.

References

1. S. Das, A. Roy, A. K. Barui, M. M. A. Alabbasi, M. Kuncha, R. Sistla, B. Sreedhar and C. R. Patra, *Nanoscale*, 2020, **12**, 7604-7621.
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