Electronic Supplementary Material (ESI) for RSC Advances.

Nano-curcumin influences blue light photodynamic therapy for restraining glioblastoma stem cells growth

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1. Experimental

Chemicals:

Bovine Serum Albumin (BSA) was purchased from sigma life science (cat# A2153), Ethanol molecular grade was purchased from (Merck 1.00983.0511). MTT dye (cat#TC191), Glutaraldehyde 25% w/v solution (cat #RM5927), streptomycin penicillin solution and MANNITOL were procured from HI-media. DMEM, FBS, Trypsin EDTA, Curcumin and dialysis membrane were purchased from sigma Aldrich.

Characterization

X-ray diffractograms (XRD) were recorded on a Bruker powder XRD D8 X-ray diffractometer. Field Emission Scanning Electron Microscopy (FESEM) images were obtained with JEOL JSM-7600F at 5kV. Fourier transform infrared (FTIR) spectra were recorded on Cary Agilent 660 IR spectrophotometer. For each spectrum, 256 scans and 4 cm^{-1} resolution was applied over the range of 400–4000cm^{-1}. Micro titer plate reader BioTek synergy 2, finland was used to record the absorbance and fluorescence change of BSA and curcumin interactions. CD spectra were recorded using JASCO J-1500 Circular diachroism spectrophotometer, Easton, MD, USA using demountable cells (0.1 mm path length, Hellma). Size and zeta potential measurement were performed using Malvern particle size analyzer with the backscattering angle of 173°. UV-Vis spectrophotometer Shimadzu UV-2600 was used to prepare standard curve of drug and to analyze the amount of drug left in supernatant. Samples were freeze dried using lyophilizer model no. at -140°C and 10mT. Thermogravemetric analyses of the samples were performed using Perkin Elmer STA 8000.

Synthesis of Placebo BSA Nanoparticles

BSA nanoparticles were prepared by using desolvation method. 20mg/ml of BSA was dissolved in miliQ water. Drop wise addition of 90% ethanol was done under constant stirring of 700rpm at room temperature (25°C). The pH of the solution was maintained at 8.2. Addition of ethanol cause protein

precipitation marked by presence of turbidity as soon as protein gets phase separated. Protein nanoparticles further crosslinked by 8% Glutaraldehyde solution in water and the solution kept on rotary shaker overnight. After overnight shaking of nanoparticles with glutaraldehyde, ethanol was vaporized using rotavapor which allow the nanoparticles to remain suspended in water phase. Nanoparticle solution was centrifuged at 30,000X g for 30 min to separate the particles. Nanoparticles were resuspended in water using bath sonicator and again centrifuged. This step repeated three times to remove excess of glutaraldehyde and unconverted BSA molecules. After washing particles were freeze dried using 5% mannitol as a cryprotectant.[1]

Drug encapsulation and loading

Curcumin was dissolved in minimal amount of ethanol and added dropwise to the 2% BSA solution in the mass ratio of 1:5. This reaction allowed to stirred for 2 hrs in the dark condition. After 2 hrs, same amount of ethanol was added drop wise as in the placebo BSA nanoparticles. Reaction of Curcumin loading was performed in dark condition to avoid the degradation of curcumin in presence of light. After washing, drug loaded nanoparticles were freeze dried using 5% mannitol as a cryoprotectant.

Drug loading efficiency and Drug encapsulation efficiency:

Drug loading efficiency and encapsulation efficiency was calculated using UV-Vis spectroscopy. Supernatant collected after centrifugation was analyzed to check the availability of the drug in the nanoparticles. Absorbance of curcumin was checked after diluting the supernatant in ethanol and calculation of the presence was done using standard curve of curcumin prepared in ethanol. After freeze drying, nanoformulation was weighed and loading efficiency was calculated which was found to be 30% with the 98.5±0.5 drug encapsulation efficiency.

$$\text{Drug encapsulation efficiency (\%)} = \frac{\text{Amount of the drug added} - \text{Amount of drug in supernatant}}{\text{Amount of Drug added}} \times 100$$

$$\text{Drug loading efficiency (\%)} = \frac{\text{Amount of drug added} - \text{Amount of drug in supernatant}}{\text{Weight of Nanoparticles}} \times 100$$

CD analysis:

CD spectra of BSA, BSA nanoparticle and drug loaded nanoparticles were recorded with a CD spectrophotometer. A quartz cell with a path length of (0.1 cm) was used in nitrogen atmosphere for measurements in the far-UV region (195-260 nm). BSA concentration was kept constant (2.5 µM), while

concentration of BSA nanoparticles (20 µM) and drug loaded nanoparticles (45 µM) was used. An accumulation of three scans with a scan speed of 50 nm/min was performed, and data were collected for each nm from 260 to 195 nm. Sample temperature was maintained at 25 °C using a Neslab RTE-111 circulating water bath connected to the water-jacketed quartz cuvettes. Secondary structure analysis was done using JASCO secondary structure analysis software.

Spectral studies of BSA and Curcumin interaction:

Curcumin interaction with BSA was studied using UV-Vis spectrophotometer. BSA (10 µM) was prepared in miliQ water while curcumin stock was initially prepared in ethanol which was later diluted in miliQ water for interaction studies. Curcumin in different concentration (5, 10, 20, 40 µM) was treated with 10 µM BSA in 96 well plate and spectra was recorded using micro titer plate reader. Interaction of curcumin was further confirmed by observing fluorescence of BSA in the presence of different concentration of curcumin. All the Fluorescence studies were performed in dark color 96 well plate.

XRD analysis

Powder XRD analysis, samples were lyophilized and put to analysis. XRD analysis was performed from 2θ values of 2 to 80° with a scan rate of 0.2 scan per minute using powder XRD. Curcumin, BSANPs, drug loaded BSANPs and Physical mixture of BSANPs and curcumin was analyzed and comparative analysis was carried out for the crystallinity of the materials.

In vitro drug release studies

In vitro drug release studies were performed in 20% ethanolic solution of PBS pH 7.2 using 12KD cutoff dialysis membrane. 1 mg of drug was weighed and suspended in 1 ml of 20% ethanolic solution of PBS pH 7.2, respective amount of drug loaded nanoparticles and physical mixture of BSANPs and drug was dissolved in same solvent. Drug release was analyzed in 9ml 20% ethanolic solution of PBS pH 7.2 as a releasing media at 37° C with stirring @ 150 rpm. One ml of aliquot was taken at different time interval and sink was replaced with the same amount of solvent, aliquot was analyzed using UV-Vis spectrophotometer after taking absorbance at 450nm.

Photodynamic therapy:

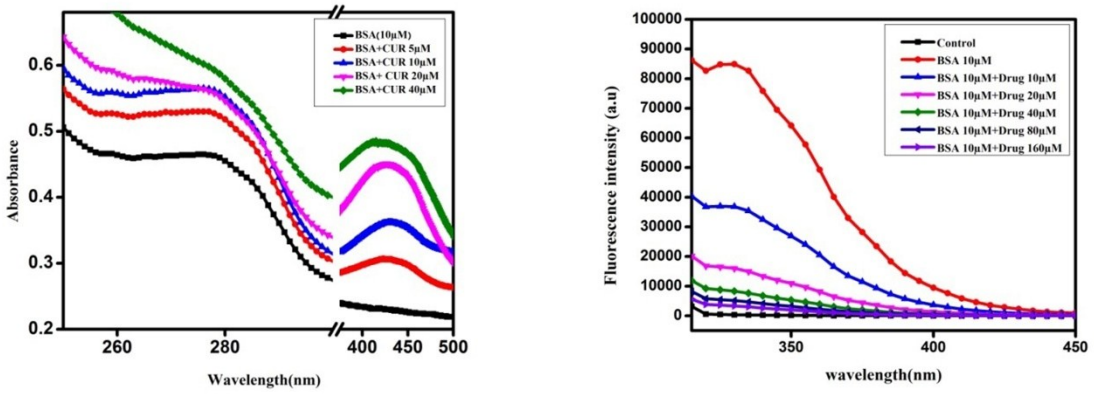
Photodynamic therapy was performed using blue led light. Blue light setup was created at INST Mohali lab. Cells were seeded in 24 well plate at the density of $1.5X\ 10^4$ on prior day to the experiment. Once cells formed monolayer, treated with different concentration of drug loaded nanoparticles, BSA nanoparticles, and drug for 2 to 6 hrs in incomplete media. After treatment cells were washed thrice

with PBS pH 7.2 and incomplete media was added. Blue light was exposed at different time interval at a fix distance of 17mm for variable time period starting from 2.5 min to 10 min exposure. After exposure cell were supplemented with 2%FBS DMEM media and incubated overnight. MTT assay was performed at 24hrs with the control that was untreated with blue light.

Table S1 Size, PDI and zeta potential data for BSANPs and Drug loaded BSANPs

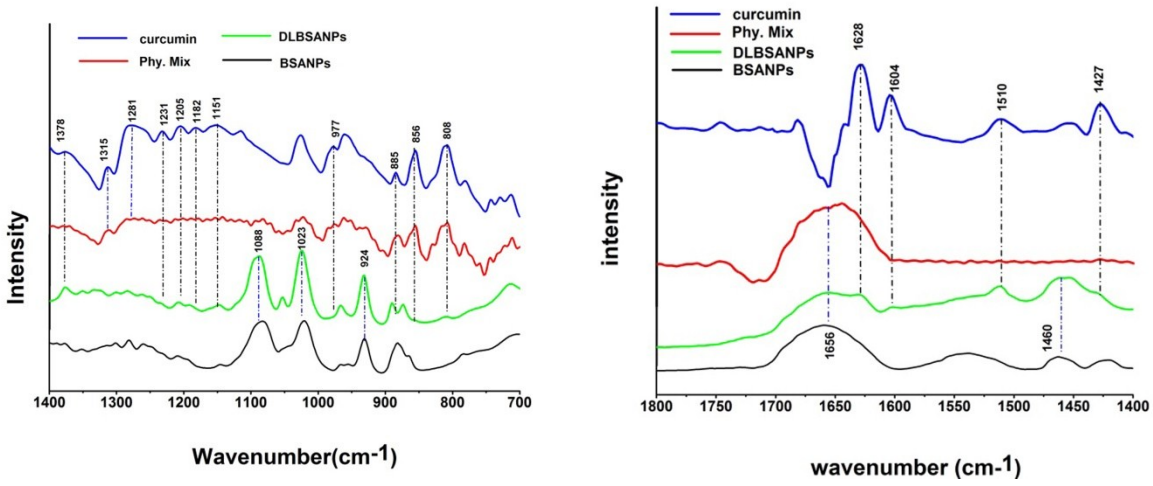
	Size Average (nm.)	PDI	Average Zeta Potential (mV)
BSANPs	106	0.058	-40.2±5.01
DLBSANPs	126	0.149	-34.5±6.73

2. Results



A **B**

Fig.S1 Interaction studies of BSA and Curcumin, Absorbacne spectra (A) fluorescence spectra (B).



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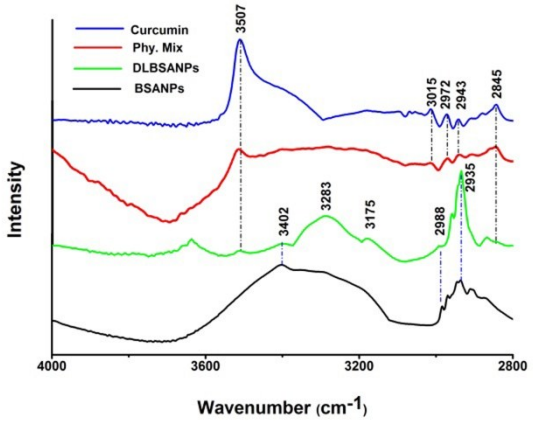
104 **(A)** **(B)**



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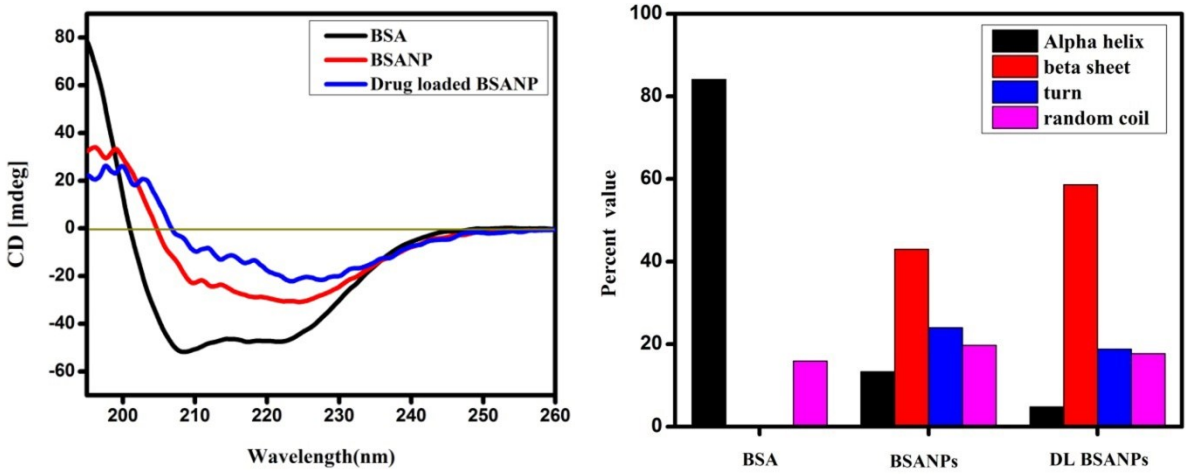
106 **(C)**

107 **Fig. S2 FTIR spectra of curcumin, BSANPs, physical mixture of BSANPs and curcumin, and**
108 **DLBSANPs showing region of interest 1400-700cm^{-1} (A), 1800-1400cm^{-1} (B), 4000-2000cm^{-1} (C).**



(A) (B)

Fig. S3 Circular Dichroism spectra of BSA, BSANP and Drug loaded BSANP (A), Secondary structure estimation in native BSA, BSANPs and DLBSANPs (B).

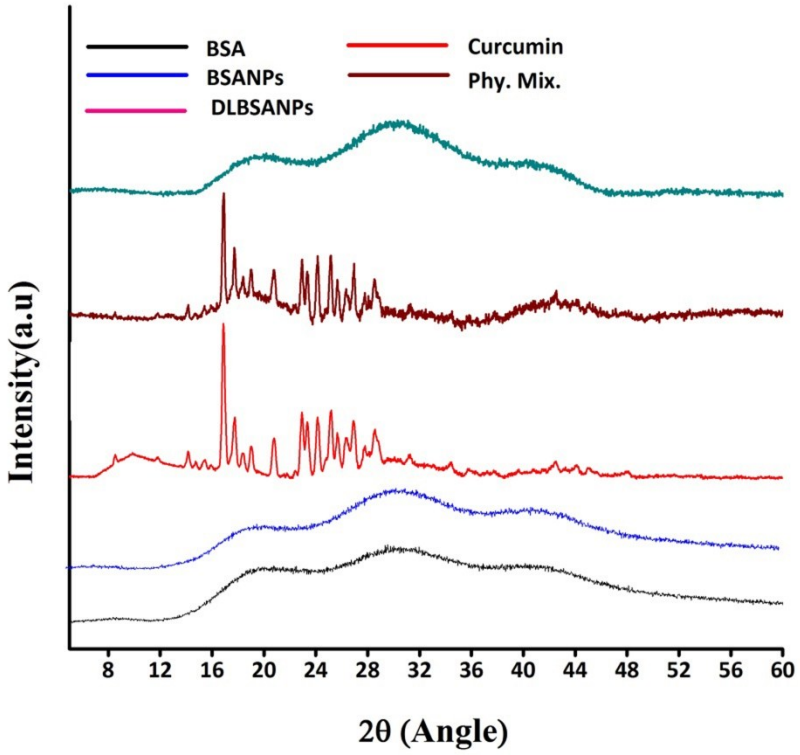


Fig. S4 XRD spectra of BSA, BSANPs, DLBSANPs, Curcumin, Physical Mixture.

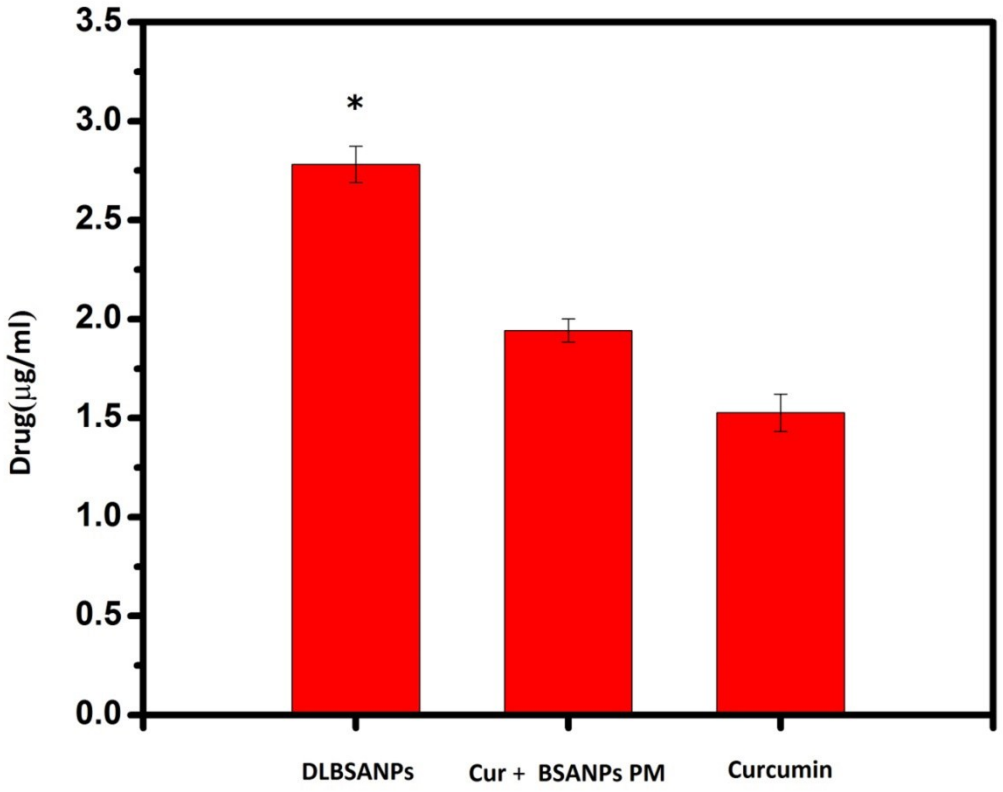


Fig. S5 *In vitro* solubility studies of Curcumin, Physical mixture of Curcumin + BSANPs and DLBSANPs.

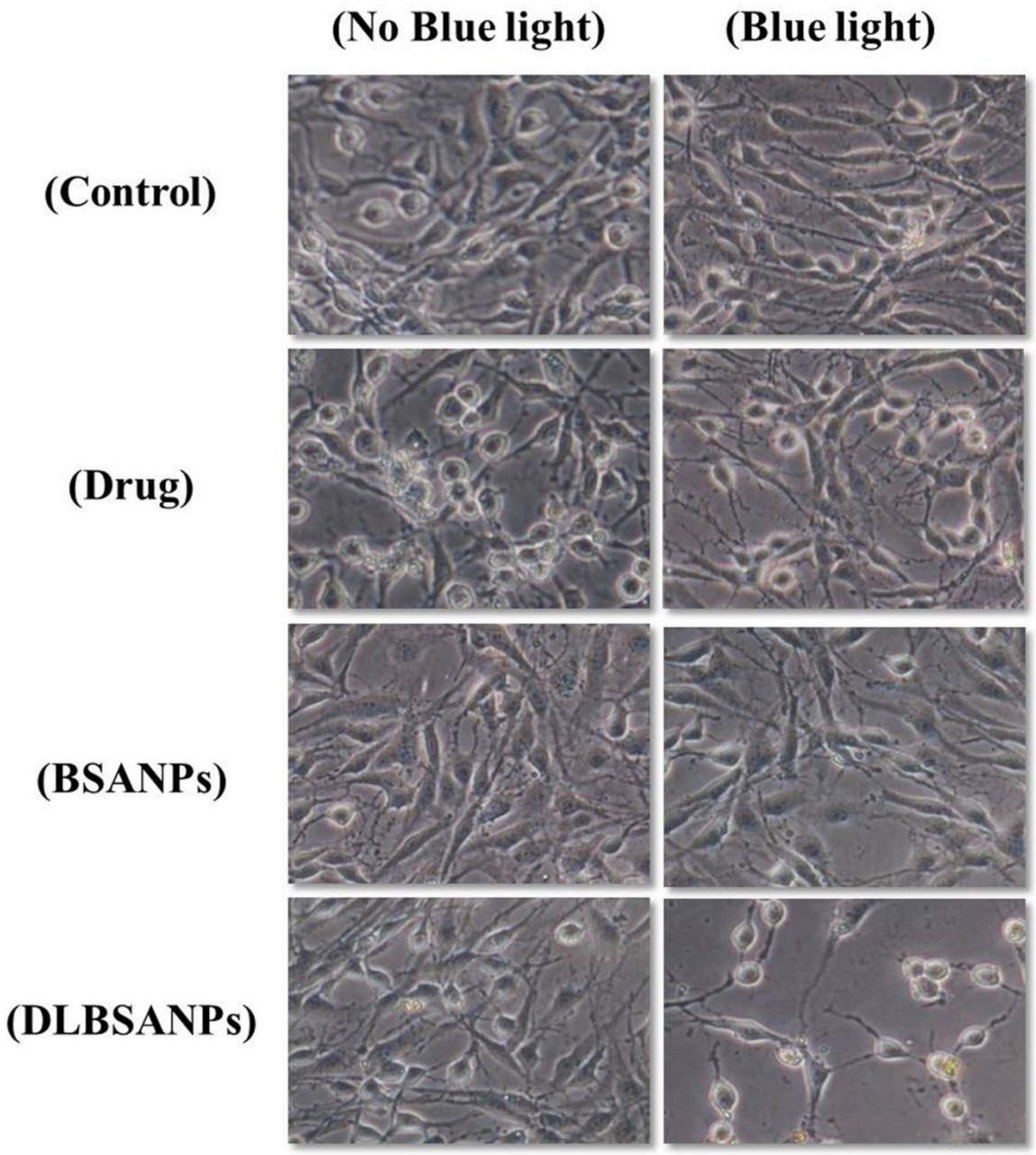


Fig. S6 Phase contrast microscopy images of C6 cell after before and after treatment of blue light for 2.5 min exposure.

References

1.D. Zhao, X. Zhao, Y. Zu, J. Li, Y. Zhang, R. Jiang and Z. Zhang, International journal of nanomedicine, 2010, 5, 669-677.