Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Bionanotherapeutics: Niclosamide Encapsulated Albumin Nanoparticles as a Novel Drug Delivery System for Cancer Therapy

Bharat Bhushan[1], Poornima Dubey[1], S. Uday Kumar[1], Abhay Sachdev[1], Ishita Matai[1], P.Gopinath[1,2] *

Nanobiotechnology Laboratory,

[1]Centre for Nanotechnology, Indian Institute of Technology Roorkee,

[2]Department of Biotechnology, Indian Institute of Technology Roorkee,

Roorkee, Uttarakhand-247667, India.

*Corresponding author: Tel. +91-1332-285650; Fax. +91-1332-273560;

E-mail: pgopifnt@iitr.ernet.in, genegopi@gmail.com

2. Materials and methods

2.1. Materials

BSA was purchased from HIMEDIA. Anticancer drug niclosamide and crosslinking agent glutaraldehyde (50 wt% in H_2O) were procured from Sigma–Aldrich and stored at appropriate storage conditions until used. The cell staining dyes Rhodamine B were purchased from life technologies. All other chemicals used were molecular biology grade. A549 cells (Non-small lung cancer cells) and MCF-7 (Breast cancer cells) were received from National Centre for Cell Science (NCCS), Pune, India. They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and 1% Penicillin-streptomycin in the 37°C incubator with 5% CO_2 and 95% air.

2.2. Preparation of BSA-Nic NPs and rhodamine conjugated BSA-Nic NPs

BSA-Nic NPs were synthesized using a desolvation method. Briefly, 5 mL of 5 mg mL^{-1} BSA solution in water was taken into a beaker under a continuous stirring on a magnetic stirrer at room temperature. Niclosamide (Stock concentration- 8 mg mL^{-1} in ethanol) were added drop wise at a constant rate of 0.8 mL/min into the above solution. The mixture was stirred for one hour followed by dropwise addition of pure ethanol till turbidity just appeared. The suspension was stirred for 10 min, followed by drop-wise addition of glutaraldehyde into it. The suspension was left for overnight stirring. Afterwards, the suspension was centrifuged at 18000 x g for 10 min in order to remove the supernatant. The residual nanoparticles were washed with ethanol to remove adsorbed glutaraldehyde and free drug from nanoparticles surface. The washed samples were resuspended in 5 mL of ultrapure water. For rhodamine conjugated BSA NPs 40μL of 10%

rhodamine solution was added with constant stirring prior to the addition of drug and rest followed the above mentioned procedure.

2.3. Characterizations of BSA-Nic NPs

FTIR spectra of BSA (control), niclosamide, BSA-Nic NPs were recorded on Thermo Nicolet FTIR spectrometer in the range 4000–400 cm^{-1} using KBr pellets. Fluorescence study conducted inside a fluorescence spectrophotometer (Hitachi F- 4600, Japan) equipped with Xenon arc lamp at an excitation wavelength of 280 nm. The excitation slit width and emission slit width being 2.5 nm and 5 nm, respectively. Dynamic light scattering (DLS, Malvern) was used to determine the mean size and surface charge of the prepared nanoparticles. Morphology and size of the particles was corroborated using FE SEM (Carl Zeiss ULTRA PLUS) and AFM (NTEGRA PNL) operating in semi-contact mode. The images were further processed using NOVA software. XRD analysis was done by using Bruker AXS D8 advance powder X-ray diffractometer (Cu-Kα radiation, λ = 1.5406A˚) in the range of 10–90º at a scan speed of 0.5º/min. Thermal studies were done by heating 10 mg of respective samples from 30ºC to 700ºC at a constant rate of 10ºC/ min in EXSTAR TG/DTA 6300 under controlled nitrogen atmosphere.

2.4. Entrapment efficiency (EE) of niclosamide

The as-prepared nanoparticles were lyophilized at−80˚C and 0.085-mbar pressure by a freeze dryer to get a lyophilized nanoparticles powder for later studies. A known amount of nanoparticles were then redispersed in ethanol and ultrasonicated for 30 minutes. After centrifugation, the resultant released drug remained in the supernatant and was analyzed by UV-

visible spectrophotometer (Lasany double-beam L1 2800) and the entrapped drug was calculated by using calibration curve of niclosamide in ethanol. The EE was calculated according to the formula given below.

$$EE = \frac{\text{Total amount of drug- Amount of drug in supernatant}}{\text{Total amount of drug}} \times 100\%$$

2.5. *In vitro* drug release studies of niclosamide from BSA-Nic NPs

The *in vitro* drug release studies were carried out by placing a 5mL of BSA-Nic NPs in a dialysis tube. The dialysis was carried out against 15 mL PBS (pH 7.4) solution in a Schott bottle, continuously shaken at 37 ^{o}C, 120 rpm. After a predefined time interval, the PBS solution was collected for analysis and replaced with equivalent amount of fresh buffer solution. The concentration of released niclosamide in PBS solution was analyzed by using an UV-visible spectrophotometer. Lastly, the cumulative percentage of drug released at a predetermined time interval was estimated from the total amount of drug loaded in BSA-Nic NPs. The percentage of drug release could be estimated from the following equation:

$$\text{Drug release [\%]} = c(t)/c(0) \times 100$$

Where, c(0) and c(t) represent the amount of entrapped drug and amount of drug released at a time t, respectively. All studies were carried out in triplicate.

2.6. *In Vitro* Stability of BSA-Nic NPs

The stability of prepared NPs in pure water and 0.9% NaCl solution was evaluated by detecting their mean diameter at 25°C by DLS. Briefly, nanoparticles were reconstituted in pure water and 0.9% NaCl at 25°C. The mean particle diameters were investigated by DLS at 0, 12, 24, 48, 72 and 96 h. To determine the stability of encapsulated niclosamide in BSA-Nic NPs, lyophilized and 0.9% saline solution of BSA-Nic NPs were kept for 0, 12, 24, 48, 72, or 96 hours at 25^{o}C. At each time point, samples were taken to find out the drug content at the beginning and after 96h incubation at 25°C. After extracting drug into ethanol, drug content was measured spectrophotometrically by using calibration curve of niclosamide in ethanol.

In-vitro anticancer activity of BSA-Nic NPs

2.7. Cell viability assay

The cell viability assay of prepared nanoparticles were evaluated on A549 and MCF-7 cells by a colorimetric assay, also known as 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. It depends on the selective capability of viable cells to form purple colored insoluble formazan crystals, a reduced product of tetrazolium component of MTT. Briefly, 10^4 cells were seeded per well in a 96 well plate. Different concentration of drug loaded BSA NPs and bare BSA NPs were formed after diluting with the media. Once the cells were attached, they were treated with above said nanoparticles and incubated at 37 ºC in incubator with 5% CO_2 for 24 hours. After 24 hours, the spent media was removed and cells were given a brief PBS wash. Fresh media (DMEM) (~ 90µL) containing 10µL of MTT (stock concentration-5mg/mL in PBS) was added to each well. The cells were incubated at 5% CO2 incubator for 3-4 h, insoluble formazan crystals was formed by mitochondrial dehydrogenases enzyme. The supernatant spent

media was removed and formed crystals were solubilized in dimethyl sulfoxide (DMSO) and incubated at room temperature over gyratory shaker for 15-30 minutes until the all formazon crystals get solubilized. The optical density of the solution was taken at a wavelength of 570 nm and 690 nm using a Cytation 3 cell imaging multi mode plate reader (Biotek). Samples were analyzed in triplicate for each experiment. For drug alone study DMSO is used as solvent for niclosamide and equivalent amount of DMSO alone is taken as reference.

2.8. AO/EB Staining

In order to differentiate apoptotic nuclei from necrotic nuclei, the BSA-Nic NPs treated cells were stained with a combination of AO/EB dye. Cells were seeded in 6-well plates and left overnight for attachment, followed by the treatment with desired concentration of BSA-Nic NPs. After 24 h of incubation, media were removed and the cells were washed thoroughly with PBS followed by the addition of fresh medium containing 10 µL of AO/EB mix (10 mg mL^{-1} AO and 10 mg mL^{-1} EB stock solution in PBS) in each well. The cells were incubated at 37°C for 5-10 minutes and then given a PBS wash to remove the excess dyes (to avoid background fluorescence of free dye) before visualizing under a EVOS cell imaging system (Life technologies, USA) and images were captured under blue filter, green filter.

2.9. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

For gene expression studies, 2 x 10^5 cells were seeded in a 3.5 cm plate and grown for 24 h, followed by subsequent treatment with desired concentrations of BSA-Nic NPs. Thereafter, Tri reagent (Sigma-Aldrich, USA) was used to isolate the total RNA from entire cell population. Reverse transcription was performed in order to generate cDNA from total denatured RNA (1

μg) at 42 ^{o}C for 50 min via Superscript II Reverse Transcriptase (Invitrogen, USA) in a total reaction mixture of 20 μL. Expression of apoptotic signaling genes were examined by RT-PCR analysis using housekeeping gene β actin as internal control. Semiquantitative PCR was carried out with 1 μL (5 times diluted stock of the above RT product) along with the gene-specific upstream and downstream primers in Veriti 96 well thermal cycler (Applied Biosystems). Initial denaturation temperature was set at 94 ^{o}C for 3 min, followed by a PCR cycle of (i) denaturation at 94 ^{o}C for 30 s, (ii) annealing at 60 ^{o}C for 30 s, (iii) extension at 72 ^{o}C for 1 min and finally a extension at 72 ^{o}C for 10min. Finally, the PCR products were analyzed on 1.2% agarose gel and band intensity was measured via Gel Doc (Bio-Rad).

2.10. FE-SEM analysis of cell morphology

Cells were seeded over glass cover slips in a 6- well plate and then treated with desired concentrations of BSA-Nic NPs for 24 h. The medium was removed and cells were washed with PBS. Thereafter, cells were fixed with 2% glutaraldehyde solution, followed by the dehydration in graded ethanol solutions and finally the samples were air dried. Finally, the sputtered gold coating of attached cells was done for observation under FE-SEM.

2.11. Cell uptake studies

Briefly, A549 and MCF-7 cells were grown in 6 well plate with a density of 2 x 10^5 cells per well and then incubated at 37 ºC in incubator with 5% CO_2. After overnight incubation, the spent media was removed, followed by PBS wash. Then the cells were incubated with rhodamine conjugated BSA-Nic NPs for 8-12 hours. Thereafter media was removed and cells were given a

brief PBS wash. The cells were then viewed under EVOS cell imaging system (Life technologies, USA) and images were captured under green filter and transmitted mode.

References

1. L. Zheng, H. Jungheun, Z. Tao and G. Liwei, *Food Funct*., 2014, **6**,1278-1285.
2. S. Sripriyalakshmi, C. H. Anjali, C. G. P. Doss, B. Rajith and A. Ravindran, *PLoS ONE*., 2014, **9**, e86317.
3. D. Zhao, X. Zho, Y. Zu, J. Li, Y. Zhang, R. Jiang, and Z. Zhang, *Int. J. Nanomed.,* 2010, **5**, 669-677.
4. Y. Zu, Y. Zhang, X. Zhao, Q. Zhang, Y. Liu and R. Jiang, *Int. J. Nanomed.,* 2009, **4**, 321-333.
5. L. Zhao, R. Su, W. Cui, Y. Shi, L. Liu and C. Su, *Int. J. Nanomed.,* 2014, **9**, 2149-2156.
6. A. V. Jithan, K. Madhavi, M. Madhavi and K. Prabhakar, *Int. J. Pharm. Investig.,* 2011, **2**, 119–125.

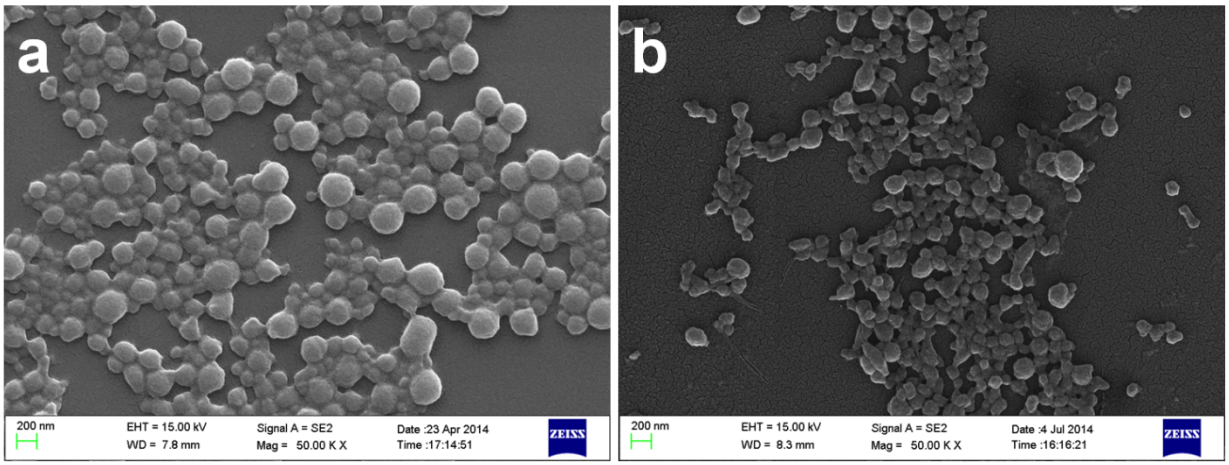

Fig. S1 FE-SEM image of (a) BSA NPs and (b) rhodamine conjugated BSA-Nic NPs, showing their typical morphology. (Scale bar- 200nm)

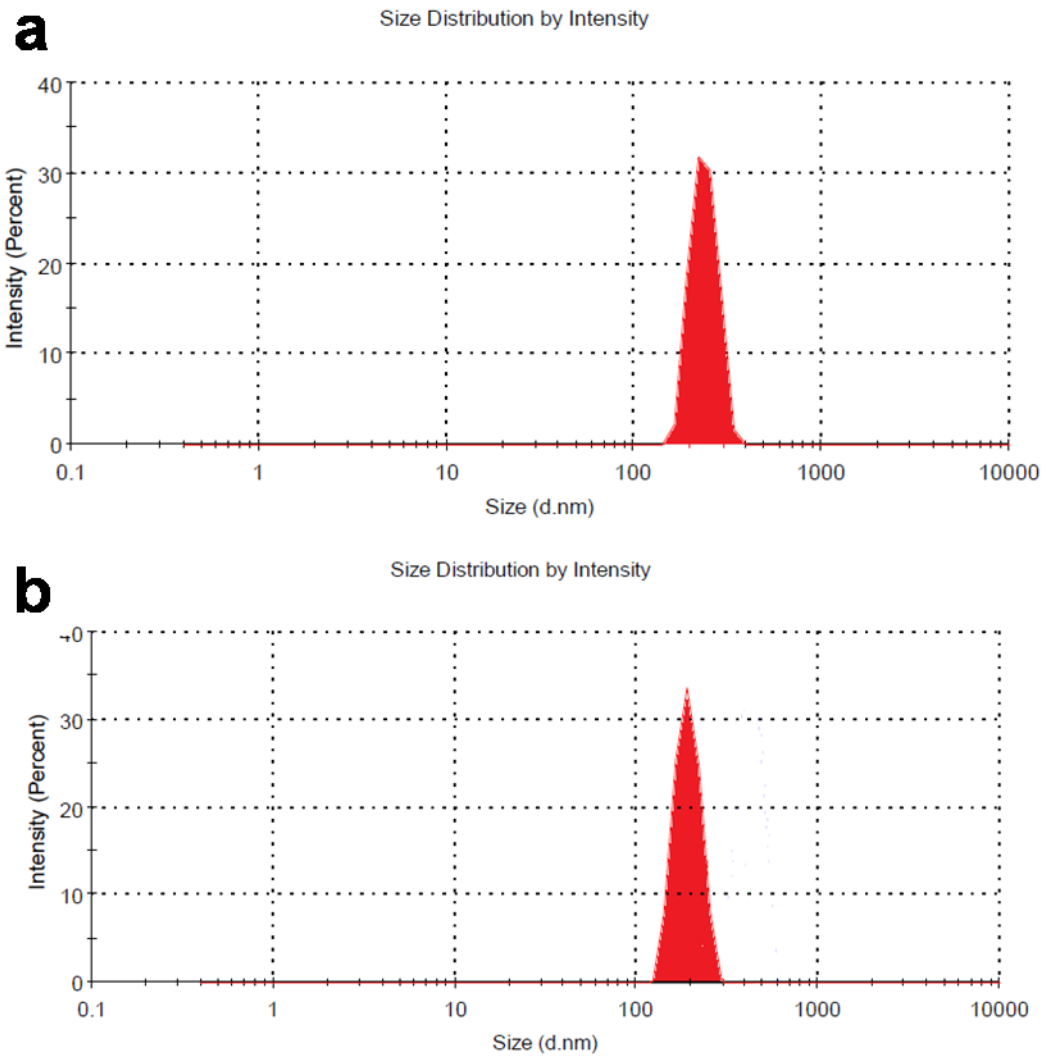

Fig. S2 DLS images of (a) BSA nanoparticles (b) rhodamine conjugated BSA nanoparticles.

Characteristics	**BSA-Nic NPs**	**BSA-EGCG**	**BSA-ATV**	**BSA-PTX**	**BSA-VBLS**	**BSA-LTB-5-FU**	**BSA-Cur**
Morphology	spherical	spherical	spherical	spherical	spherical	spherical	NM
Size (nm)	200	186-300	97–125	210	93-282	254	223.5-228.7
Entrapment efficiency (%)	75.96-92.36	NM	71-94	95.3	84.83-94.78	80.1	74.76-91.01
Preparation technique	desolvation	desolvation	desolvation	desolvation	desolvation	desolvation	desolvation
Zeta potential (mV)	-34.2	-11.5--42.9	-36.3	-30	NM	-19.95	-30.1--32.2
Cell line	A549, MCF-7	Caco-2	MiaPaCa-2	PC-3	In silico	SMMC-7721	MDAMB231
Reference	Current study	Zheng et al. 2014	Sripriyalakshmi et al. 2014	Zhao et al. 2010	Zu et al. 2009	Zhao et al. 2014	Jithan et al. 2011

Vinblastine sulfate (VBLS), Niclosamide (Nic), Paclitaxel (PTX), Epigallocatechin gallate (EGCG), Heat-labile enterotoxin subunit B (LTB), 5-fluorouracil (5-FU), Atorvastatin (ATV), Curcumin (Cur), Not mentioned (NM)

Table S1. Comparison of prepared BSA-Nic NPs with previously reported drug encapsulated BSA based NPs.

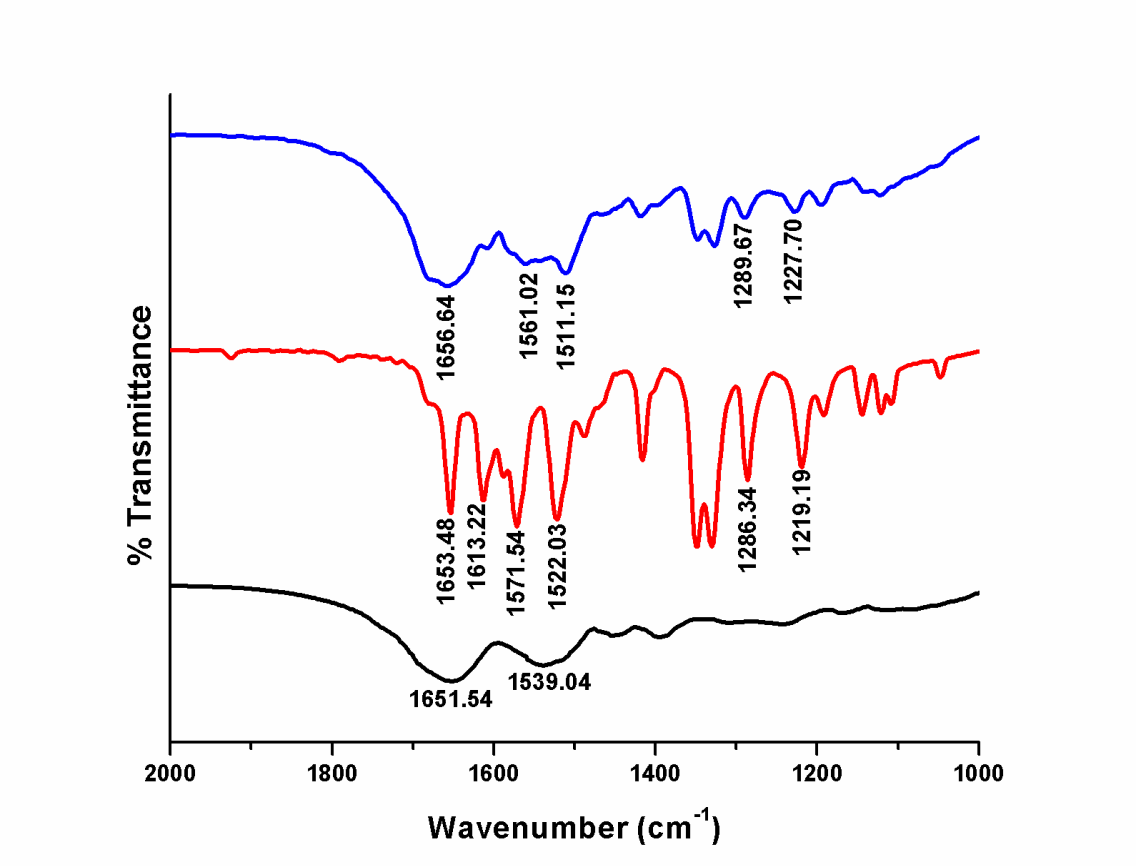

Fig. S3 FTIR spectra of BSA (control), raw niclosamide powder and BSA Nic NPs.

Functional groups	C=C	C=O	N-H	C-N	NO2	C=O	C-OH
Wave no	1613.22	1653.48	1571.54	1286.34	1522.03	1219.19	1192.09
vibration	C=C stretch	C=O stretch	N-H bend	C-N stretch	NO2 stretch	C=O stretch	C-OH stretch

Table S2. Characteristic major absorption bands in the IR spectra of the raw niclosamide powder.

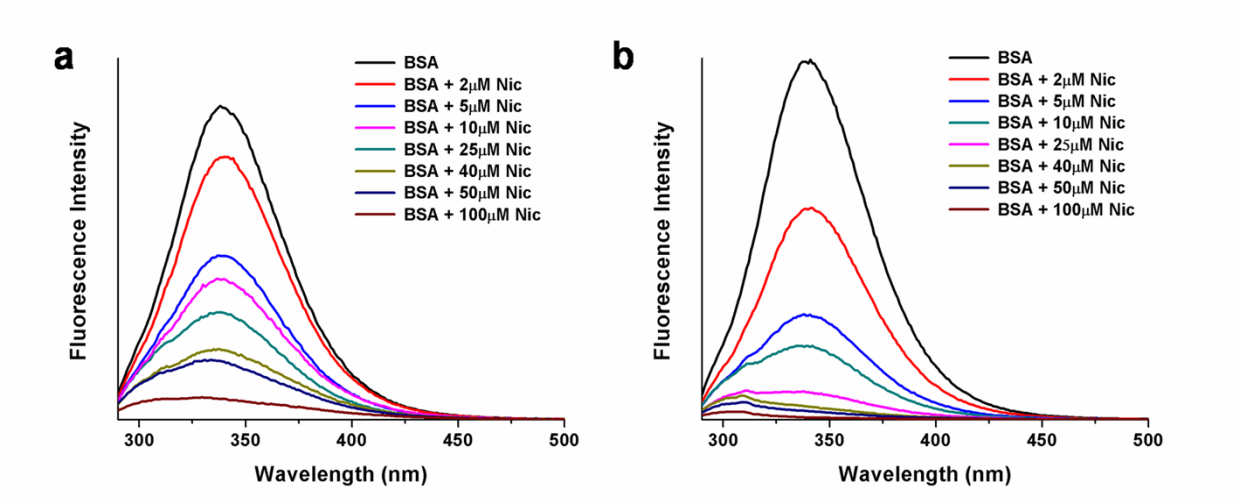

Fig. S4 Fluorescence spectra of BSA in (a) aqueous (b) PBS with different concentration of niclosamide (0-100μM). (T= 298 K, λex = 280 nm, λem= 295-500 nm).

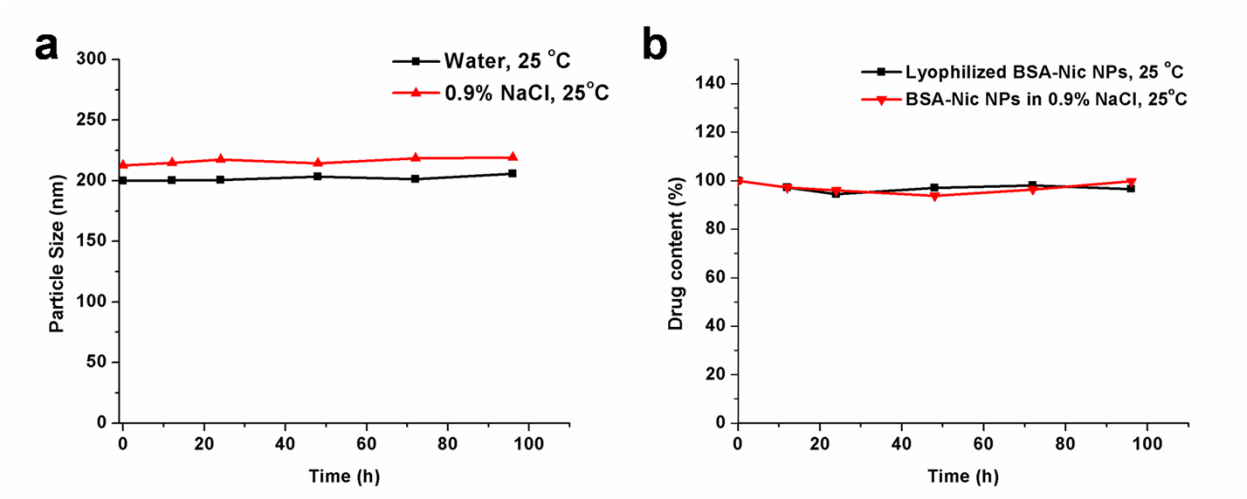

Fig. S5 Stability studies (a) size of BSA-Nic NPs in aqueous and 0.9% saline solution (b) drug content percentage (initial drug content taken as 100%)

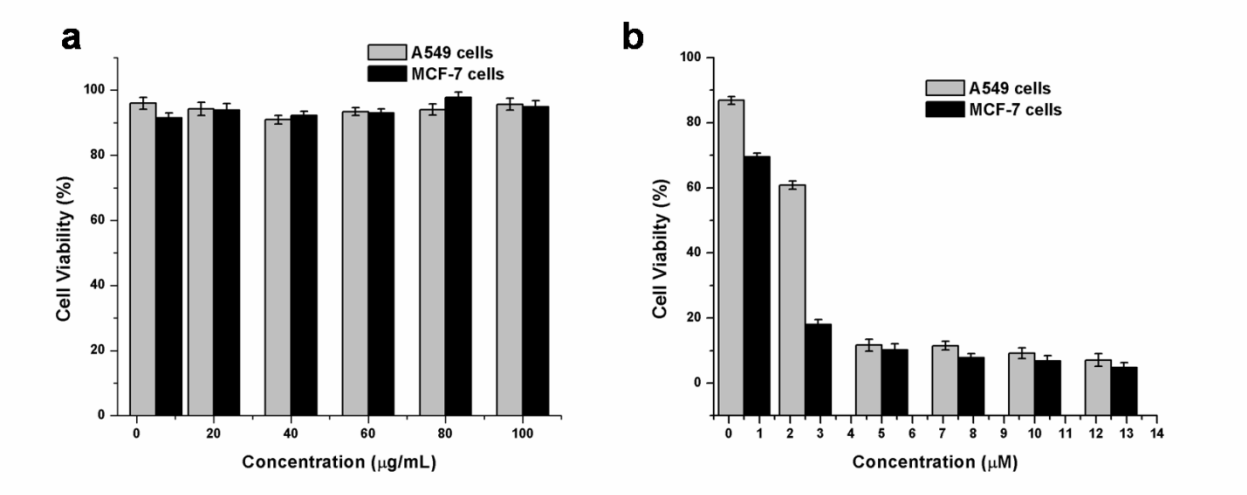

Fig. S6 Cell viability assay (MTT assay) of (a) BSA NPs and (b) niclosamide (in DMSO) on A549 cells and MCF-7 cells

Gene	Primers
Beta-actin	Forward: 5’ CTGTCTGGCGGCACCACCAT 3’ Reverse : 5’ GCAACTAAGTCATAGTCCGC 3’
p53	Forward: 5’ TGGCCCCTCCTCAGCATCTTAT 3’ Reverse : 5’ GTTGGGCAGTGCTCGCTTAGTG 3’
Caspase-3	Forward : 5’ TTCAGAGGGGATCGTTGTAGAAGTC 3’ Reverse : 5’ CAAGCTTGTCGGCATACTGTTTCAG 3’
C-myc	Forward : 5’ CCAGGACTGTATGTGGAGCG 3’ Reverse : 5’ CTTGAGGACCAGTGGGCTGT 3’
Bax	Forward : 5’ AAGCTGAGCGAGTGTCTCAAGCGC 3’ Reverse : 5’ TCCCGCCACAAAGATGGTCACG 3’
Bad	Forward : 5’ CCTTTAAGAAGGGACTTCCTCGCC 3’ Reverse : 5’ACTTCCGATGGGACCAAGCCTTCC 3’
Bcl-xl	Forward : 5’ATGGCAGCAGTAAAGCAAGC 3’ Reverse : 5’ CGGAAGAGTTCATTCACTACCTGT 3’

Table S3. List of apoptotic signaling genes primers used in semi-quantitative RT-PCR analysis.

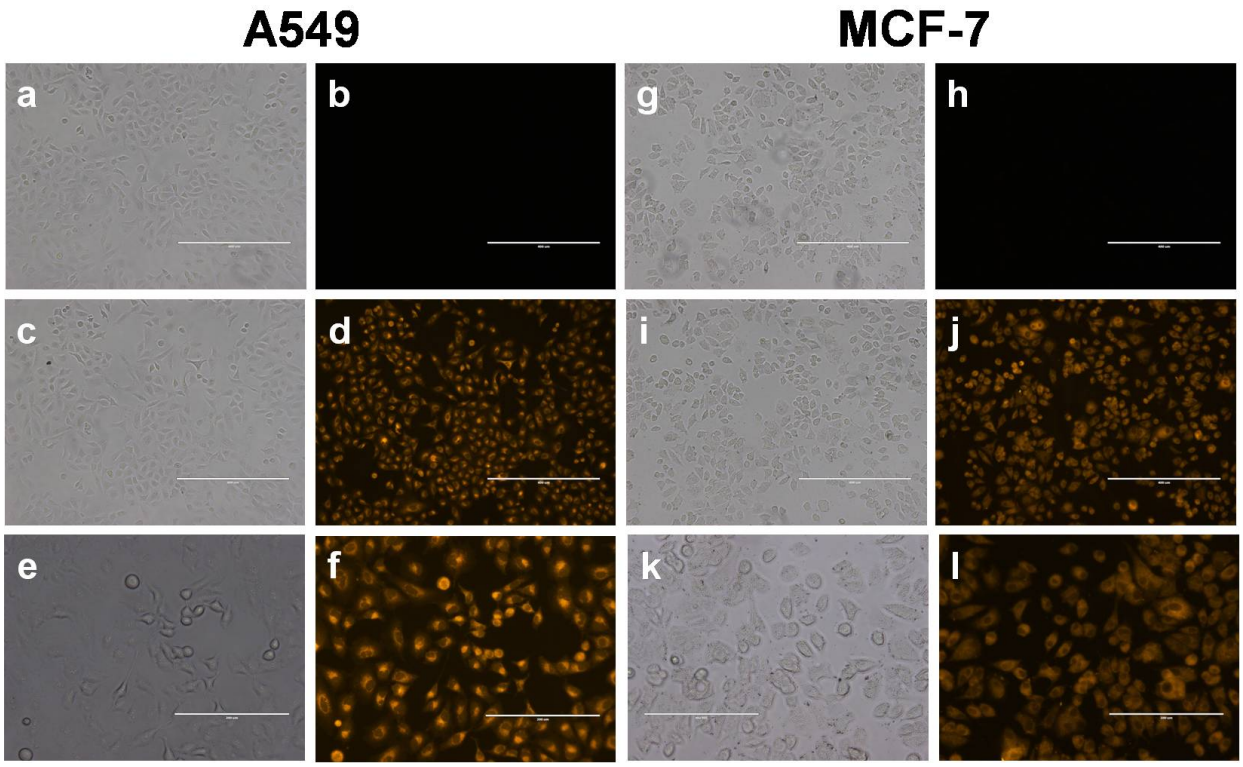

Fig. S7 Cell uptake studies of BSA-Nic NPs on (a-f) A549 and (g-l) MCF-7 cells at different magnification. Bright field (a and g) and fluorescent images (b and h) of untreated cells did not show any fluorescence. Bright field (c, i, e and k) and fluorescent images (d, j, f and l) of rhodamine conjugated BSA-Nic NPs treated cells. (c, d, I and j) at scale bar- 400 µm and (e, f, k and l) at scale bar- 200µm.