Fluorescent Magnesium Nanocomplex in Protein Scaffold for Cell Nuclei Imaging Application

Alok Pandya[a], Apritam Tripathi[a], Rahul Purohit[a], Sanjay Singh[a], Manjula I. Nandasiri[c], Ajay Karakoti[a,b] , Surinder P. Singh[b]* and Rishi Shanker[a]*

[a]Institute of Life Sciences, School of Science and Technology, Ahmedabad University,

[b]Institute of Engineering and Technology, School of Science and Technology, Ahmedabad University, Ahmedabad-380009, INDIA

Ahmedabad-380009, INDIA

[c] National Physical Laboratory, Dr. K. S. Krishnan Marg, New Delhi – 110012, INDIA

[d]Environmental and Molecular Sciences Laboratory, PNNL, Richland WA - 99354

1. Materials and methods:

1.1 Reagents:

All chemicals used were of analytical grade. Dulbecco's modified eagle's medium (DMEM), trypsin-EDTA,Dimethylsulfoxide(DMSO),3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetra-zolium bromide (MTT), Magnesium Chloride($MgCl_2$),Bovine serum albumin(BSA) and Sodium hydroxide pellets were purchased from Hi-Media (Milwaukee, USA). In all preparations, high purity deionized water from a Millipore system was used.

1.2 Synthesis of MgNC:

All glassware used in the experiment were cleaned in a bath of freshly prepared aqua regia (HCl : HNO, 3 : 1 by v/v) and rinsed thoroughly in water prior to use. A typical synthesis is described as follows: Briefly 1 mL of 5 mM Magnesium chloride was added to 1 mL of~300 μM protein (BSA) solution. After 5 min stirring, 200 μL of 1 M NaOH was added so that pH-12 was achieved. The solution was stirred continuously at room temperature with magnetic stirrer. The reaction, during which the colourless solution slowly turned light green, was stopped after stirring for 15 h. As-prepared MgNC were purified by triple centrifugation at 14,000 r.p.m for 10 min to remove impurities. The as prepared solution was stored at 4^0C for later use.

1.3 Characterisation:

UV-Vis absorption spectra were recorded on a Bio-Tek UV-vis spectrophotometer with 10 mm quartz cell at 25 ± 0.1 °C. Fluorescence emission spectra were recorded on a Varian CARY 100 Bio fluorescence spectrophotometer, with a 10 mm quartz cell at 25 ± 0.1 °C. The

sample was air-dried and subsequently examined under an Jeol High Resolution Transmission Electron Microscope. DAPI (4,6-diamidino-2-phenylindole) was used in this optical microscopic study for nucleus labelling. 100 μgmL^{-1} DAPI was applied to the cell sample for 7 min and then completely washed three times with PBS. The cell samples were then examined by an Leica DM-2000 Fluorescence microscopy equipped with a 40 X objective lens, and halogen lamps as excitation source. DLS measurements were done with a Nano S Malvern instrument employing a 4 mW HeNe laser (λ = 633 nm). Powder X-ray diffraction (PXRD) of MgNC was performed with a D8 DISCOVER with HT module, Bruker AXS GmbH, Germany with Cu Kα radiation (1.5405 Å). FT-IR analysis were done for solid dry powder after making pellets with KBr on a Shimadzu FT-IR 8400S. FT-IR spectrophotometer operating from 400 to 4000 cm^{-1}. X-ray photoelectron spectroscopy (XPS) was performed using a Kratos Axis Ultra DLD spectrometer, which consists of a high performance Al Kα monochromatic x-ray source (1486.6 eV) and a high resolution spherical mirror analyzer. X-ray source was operated at 150 W and the emitted photoelectrons were collected at the analyzer entrance slit normal to the sample surface. The data acquisition was carried out in hybrid mode with analysis area of 700 × 300 μm. The survey spectra were recorded at pass energy of 160 eV with 0.5 eV step size and high resolution spectra were recorded at pass energy of 20 eV with step size of 0.1 eV. The pass energy 20 eV in the 700 × 300 μm analysis area is referred to the FWHM of 0.59 eV for Ag $3d_{5/2}$. The charge neutralizer with low energy electrons was used to exclude the surface charging effects and the binding energy of C 1s at 284.8 eV was used as the charge reference. The chamber pressure was maintained at ~ 5 × 10^{-9} Torr during the measurements. XPS data was analyzed by CasaXPS software using Gaussian/Lorentzian (GL(30)) line shape and Shirley background correction. The quantum yield of MgNC was determined relative to the standard fluorophore whose quantum yield was well-known (QR). The quantum yield of MgNC was determined by using the following equation.

$$\varphi_s = \varphi_r \frac{I_s A_r \eta_s^2}{I_r A_s \eta_r^2}$$

In this equation, $\boldsymbol{\varphi}$ = quantum yield, I = total integrated fluorescence intensity, A = absorbance, η = refractive index, S = Sample and R = Reference.

In this study we have used quinine sulfate in 0.5 M sulfuric acid as a standard and its quantum yield (QR) was 0.54. The quantum yield of synthesized MgNC was calculated as 0.17.

1.4 Cell Viability/Cytotoxicity Studies:

In-vitro studies were performed to characterize the biocompatibility of MgNC, for this A549 cell line (immortal cell line derived from human lung adeno-carcinoma) was purchased from the National Center for Cell Sciences (NCCS) Pune, India. Cells were maintained with Dulbecco's minimum essential medium- F12 supplemented with 10% fetal bovine serum in a humid incubator (37 °C and 5% CO_2). Cytotoxicity studies were done with sterilized(MgNC for further use of biological applications.

1.5 MTT assay:

To delineate biocompatibility of MgNC, *In-vitro* biocompatibility studies of MgNC were carried out on A549 cell line via MTT assay (Mosmann et.al, 1983). MTT assay is a quantitative colorimetric assay which assesses cell proliferation.. Cells (10^4cells/well) were plated onto 96 well plate glass. Cells were incubated with and without MgNC for ~24 h at 37 °C, in a 5% CO_2 humidified incubator followed by removal of media. An amount of 0.5 mg/mL of MTT in DMEM basal was prepared in a dark environment. After discarding the old media, 200 µL of the freshly prepared MTT solution was added to each of the cell-containing wells, followed by incubation for 4 h. After incubation, media was removed, and DMSO (200 µL) was added. The viability of cells was determined by measuring the absorbance of dissolved formazan crystals at 570 nm. All the in vitro cytotoxicity experiments were performed in triplicate, and out of it, the best three was taken to plot the MTT assay. The graph represents that MgNC do not cause any acute cytotoxicity.

1.6 Cellular uptake studies:

Cells were seeded in 6 well plate at a final concentration of 10^4 cells/ml. MgNC was added at a final concentration of 50µg/ml for 24 hrs at 37 °C at 5% CO_2. After this, cells were washed with 1X PBS buffer for two times. Cells were trypsinised with 0.25% trypsin EDTA. Cells were then centrifuged at 1200 r.p.m for 10 min at room temperature. Supernatant was discarded and cells were resuspended in 500µl 1X PBS buffer. Further forward scattering and side scattering analysis was done according to using FACS callibur flow Cytometry.

1.7 Nuclear morphological studies

To study the change in nuclear morphology cells were seeded on 18 mm coverslips at a density of 10^4 cells in a 12 well plate. Cells were incubated with 50 µg/ml of MgNC for 1h,3h,6h,12h and 24h. Media was aspirated and cells were washed twice with ice cold 1X PBS buffer. Cells were then incubated with 4% paraformaldehyde for 20 min at 4°C. Then cells were permeabalized with the 0.05% tritonX-100 and were stained with DAPI for three to five minutes. Cells were washed thrice with ice cold 1X PBS and the coverslips were mounted on antifade mounting medium and visualised under 2d- epifluorescent microscopy.

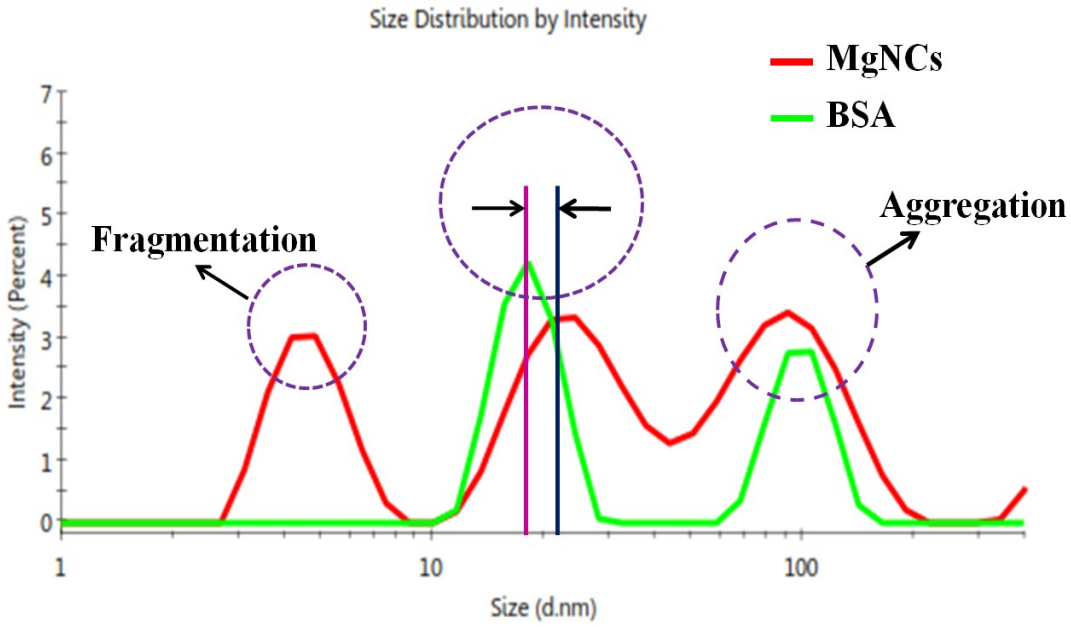

Fig.S1 DLS measurements of BSA (Green) and MgNC (Red)

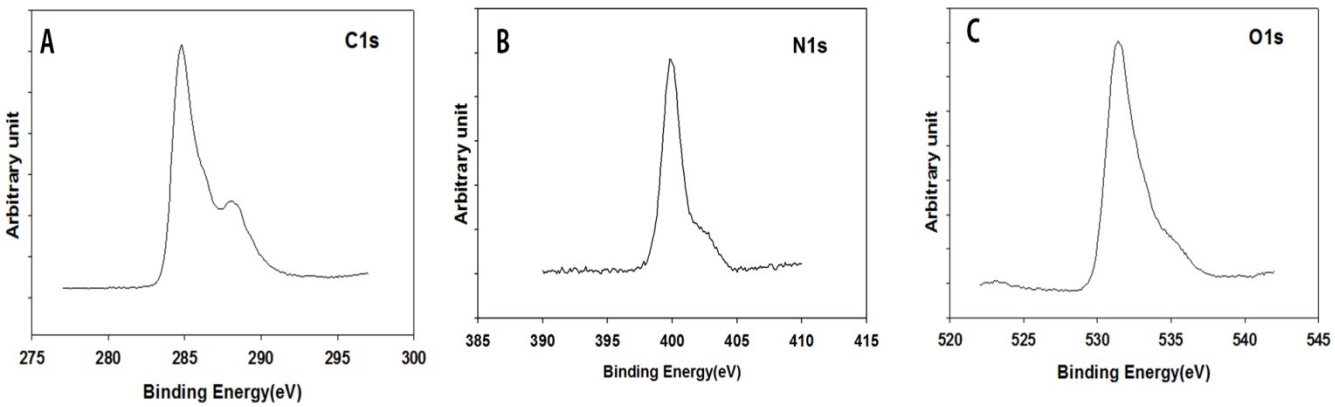

Fig. S2 XPS spectrum in the (A) C 1s, (B)O 1s and (C)N 1s region of MgNC

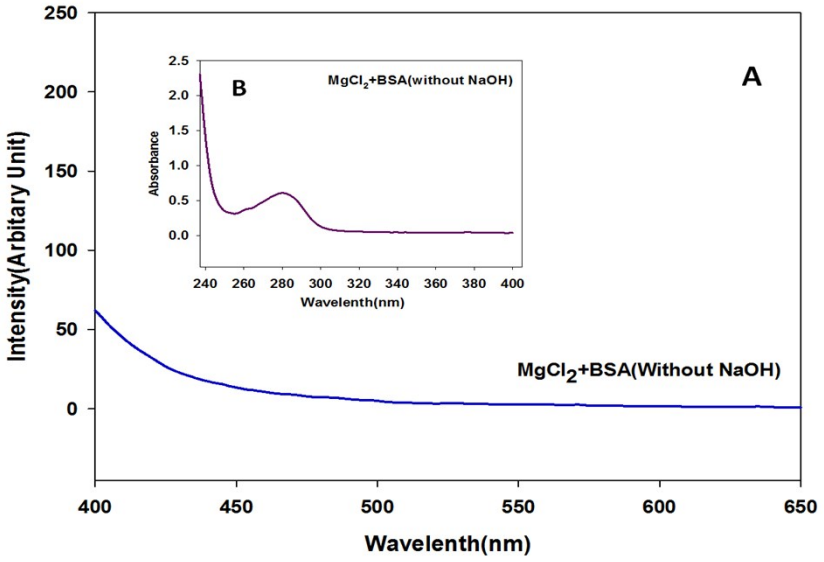

Fig. S3 The fluorescent emission spectrum (A) and UV-Vis spectrum of unreduced Mg+ BSA (without NaOH) (B)

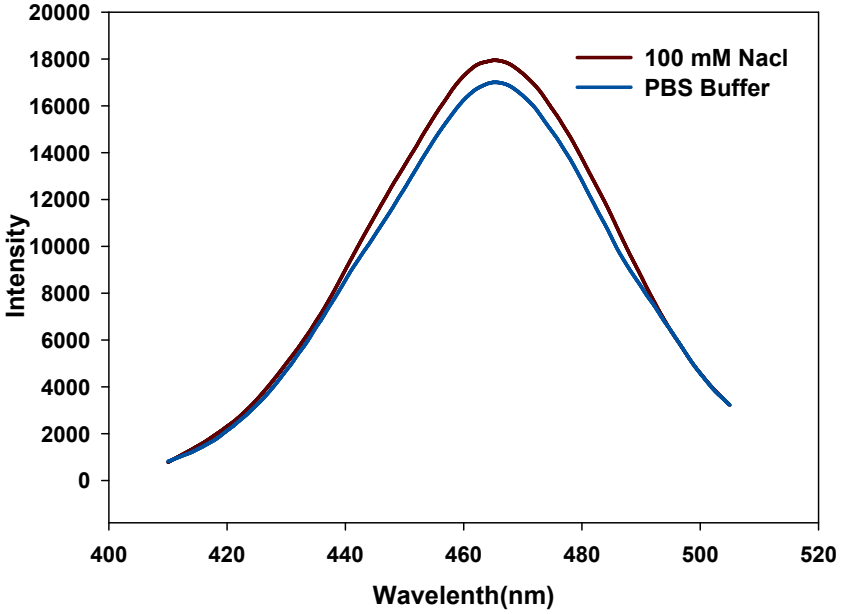

Fig. S4. The fluorescent emission spectrum of MgNC was recorded in physiological and high salt concentrations.

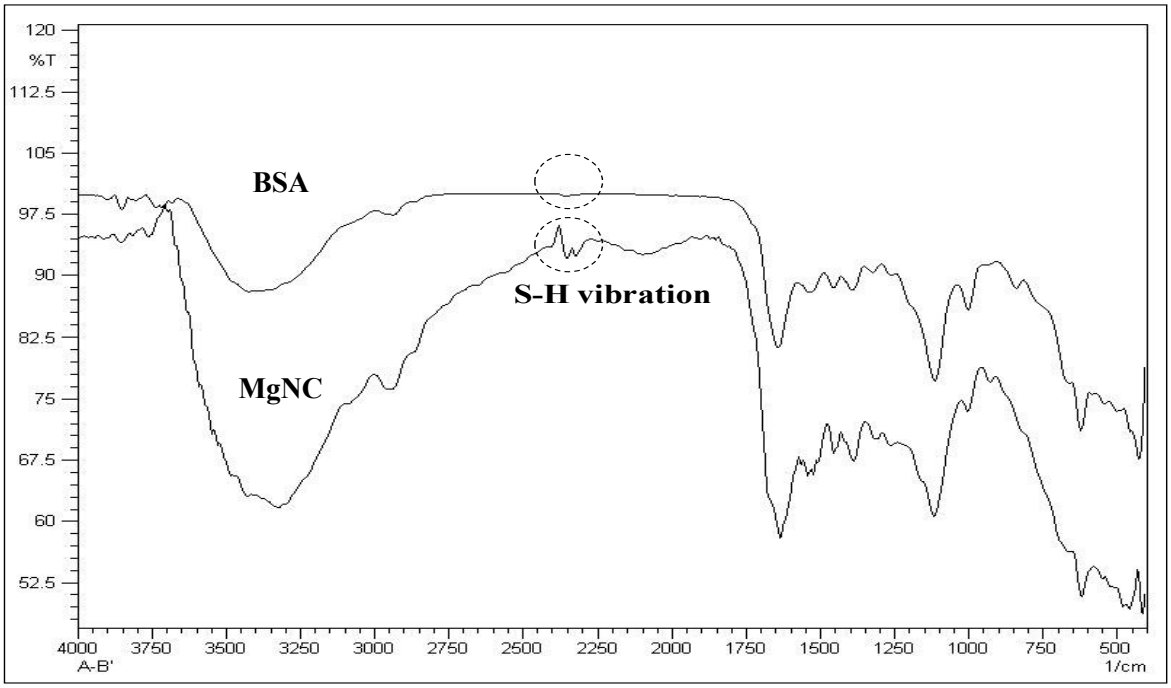

Fig. S5 FT-IR spectrum of BSA and the MgNC. The spectra clearly show the absence of S−H stretching at 2499 cm^{-1} after MgNC formation

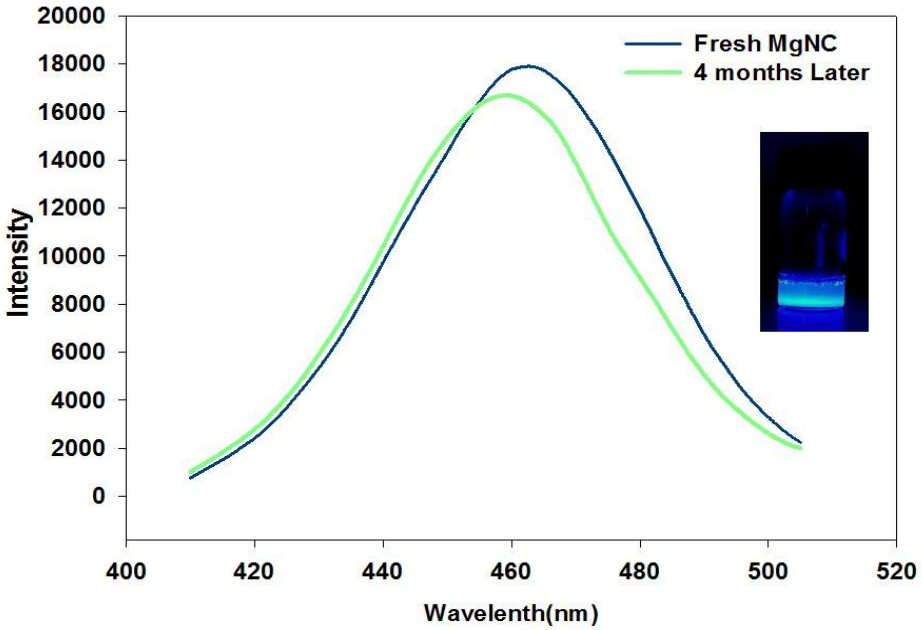

Fig.S6 Fluorescence emission spectrum of fresh and 4 months MgNC. (Inset Fluorescence of 4 months old MgNC solution under UV illumination)

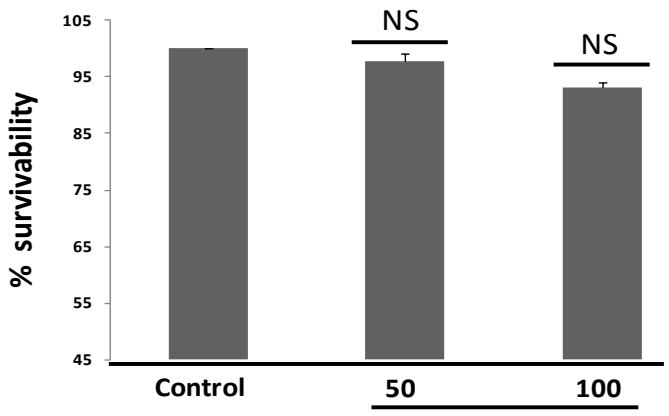

Fig.S7 MTT assay of MgNC with A549 cells

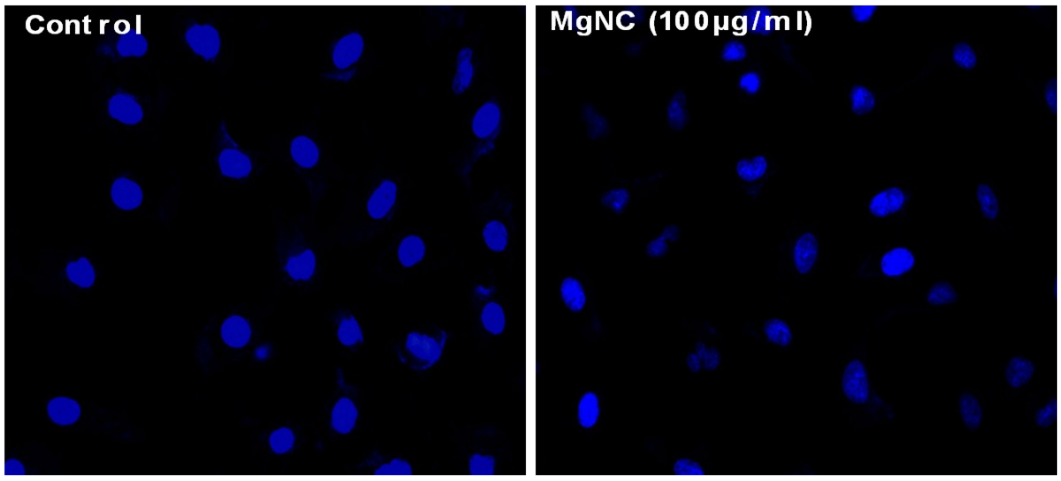

Fig.S8 Nuclear morphological analysis to effect of MgNC on apoptosis

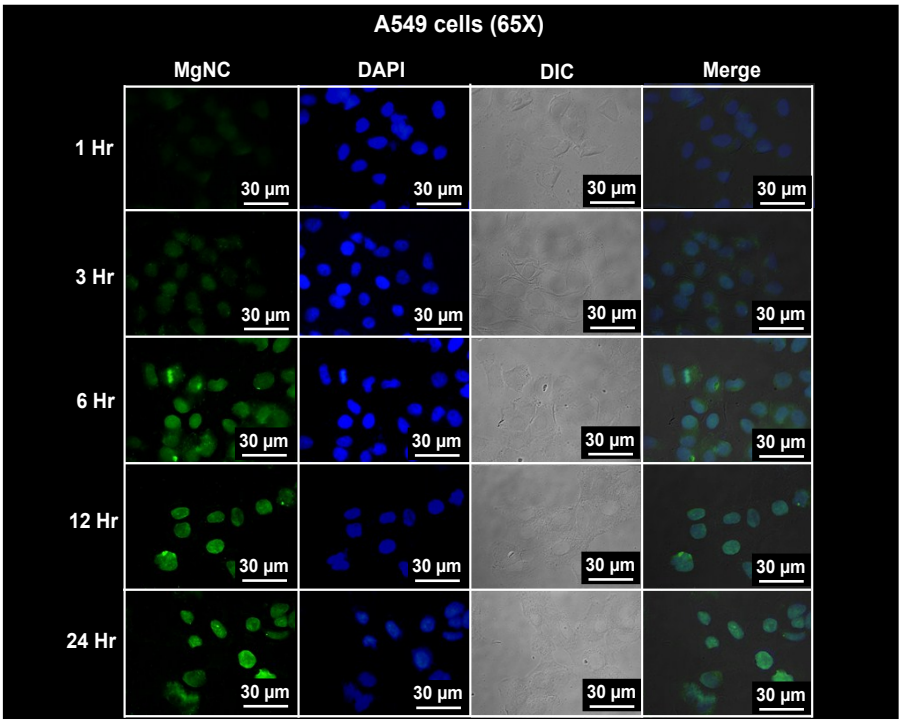

Fig.S9 : Fluorescence microscope images of A549 cells showing the time dependent uptake and co-localization of MgNC. Cells were incubated with MgNC for 1 h, 3 h ,6 h ,12h and 24 h followed by staining with DAPI

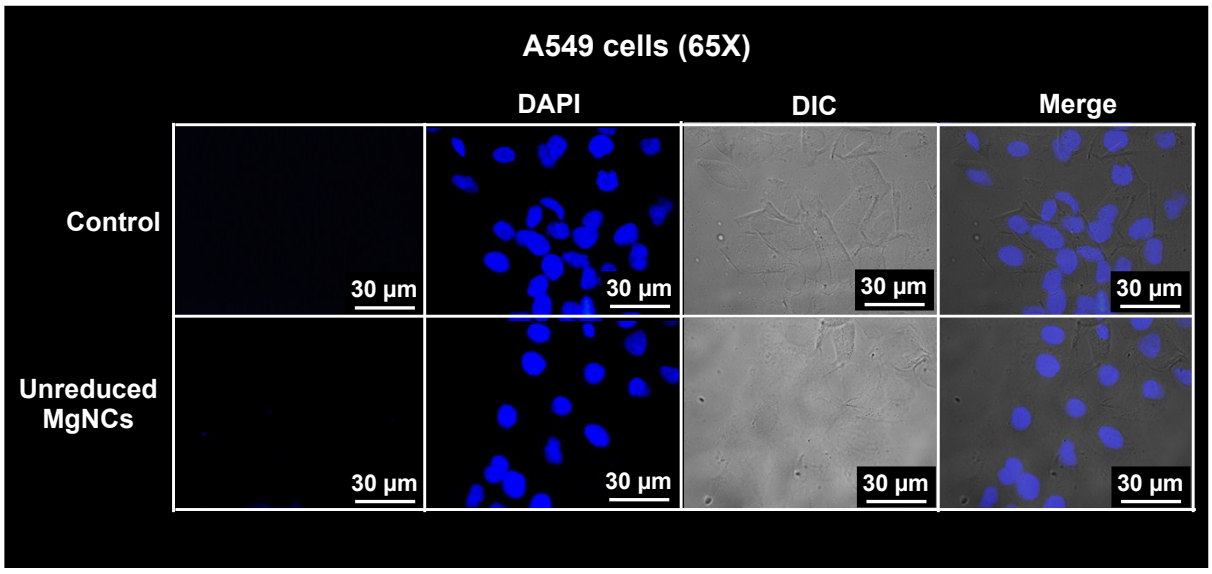

Fig.S10 Fluorescence microscope images of A549 cells incubated with Mg + BSA (without using NaOH)

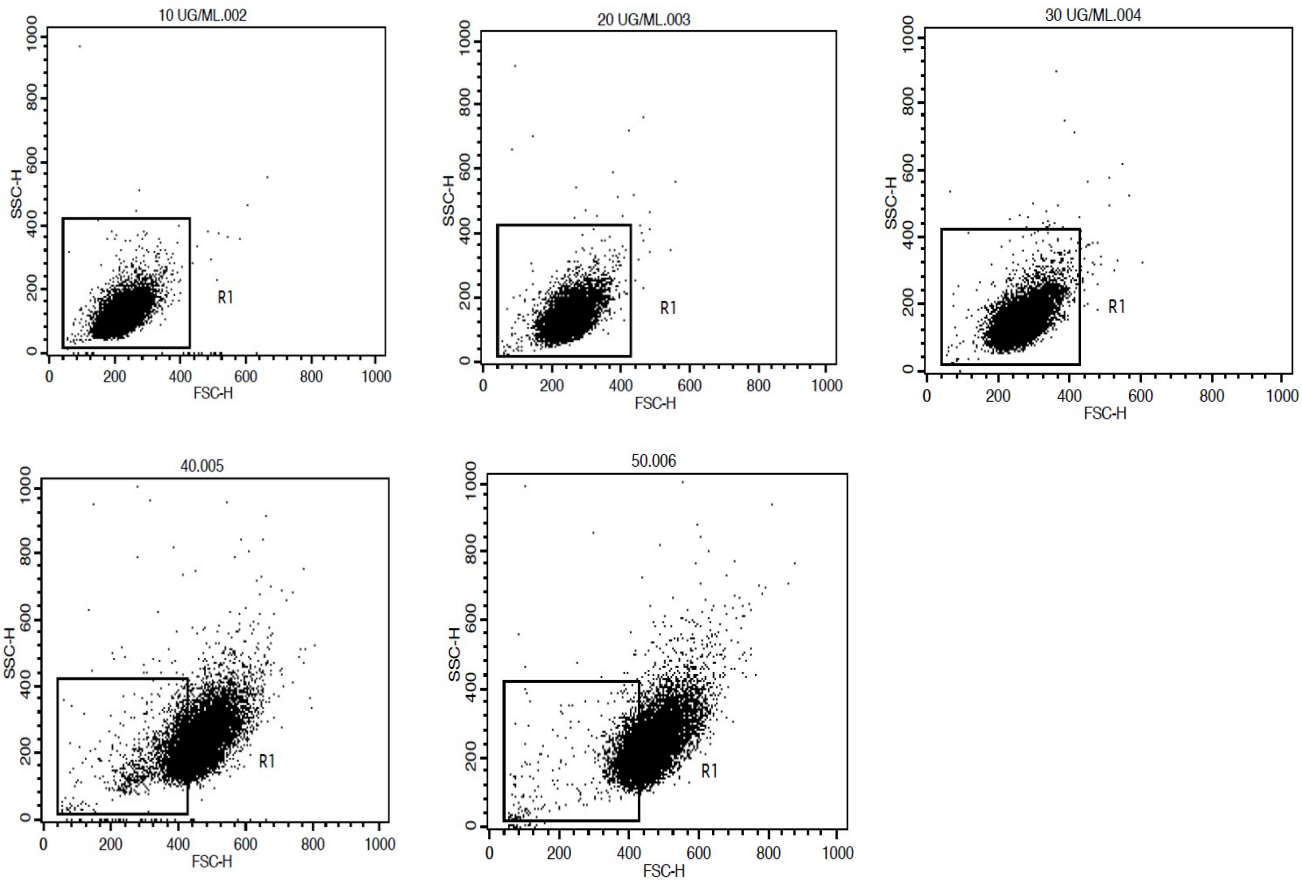

Fig.S11 Uptake study of MgNC in A549 cells using flow cytometer different concentrations (10, 20, 30, 40 and 50 µg/ml) of MgNC were exposed to A549 cells for 24 h.

References:

1. T. Mosmann, *J Immunol Methods*. 1983,**16**,65,55-63