Carbohydrate Polymers 84 (2011) 1158–1164

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Preparation, characterization, *in vitro* drug release and biological studies of curcumin loaded dextran sulphate–chitosan nanoparticles

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ARTICLE INFO

Article history:
Received 4 November 2010
Received in revised form 3 January 2011
Accepted 5 January 2011
Available online 12 January 2011

Keywords:
Chitosan
Dextran sulphate
Nanoparticles
Curcumin
Controlled delivery
Cytotoxicity
Cell uptake studies

ABSTRACT

Nanoformulation of curcumin, (a low molecular weight hydrophobic drug) was prepared by using dextran sulphate and chitosan. The developed nanoparticles were characterized by Dynamic Light Scattering measurements (DLS), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), Fourier Transform Infrared Spectroscopy (FT-IR), X-ray Diffraction (XRD) and Differential Thermal Analysis (DTA). The prepared system showed an average size of 200–220 nm with a zeta potential value of −30 mV and showed ~74% drug entrapment efficiency. *In vitro* drug release studies showed a controlled and pH dependent curcumin release over a period of one week. The cytocompatibility of bare nanoparticles was verified by MTT assay; cellular internalisation of curcumin loaded nanoparticles was confirmed by fluorescent imaging and quantified spectrophotometrically, anticancer activity of curcumin loaded nanoparticles was proved by MTT assay and reconfirmed by apoptosis assay (FACS). The results showed preferential killing of cancer cells compared to normal cells by the curcumin-loaded nanoparticles. Thus the developed curcumin loaded nanoformulation could be a promising candidate in cancer therapy.

1. Introduction

Chitosan [β-(1–4)-2-amino-2-deoxy-D-glucose], a deacetylated form of chitin is a naturally occurring linear biodegradable polysaccharide and it is made up of *N*-acetyl-D-glucosamine and D-glucosamine (Anitha et al., 2009). It is having a wide range of biological properties such as mucoadhesion (Li et al., 2008) and antibacterial property (Anitha, Deepa, et al., 2011), it can form complexes with DNA and polyanionic-polymers (Jayakumar et al., 2010), can open intercellular tight junctions (Chen, Yeh, & Chiang, 1996) and it is totally biocompatible (Jayakumar et al., 2010; Li et al., 2010).

Dextran sulphate is also a biocompatible polyanionic polymer. It is a highly branched polysaccharide with 1–6 and 1–4 glycosidic linkage with approximately 2.3 sulphate groups per glucosyl unit. It is widely used in medical field as a plasma volume extender. Several preparations of dextran were used as anticoagulants (Baba et al., 1988). In drug delivery applications many drug conjugates of dextran sulphate have been reported (Mitra, Gaur, Ghosh, & Maitra, 2001).

Dextran sulphate–chitosan NPs are formed due to the electrostatic interaction between chitosan and dextran sulphate. The surface charge is tunable by varying the ratio of the two-polymer concentrations and pH sensitive swelling has been reported (Lin, Yu, & Yang, 2005). Also the prepared NPs have good stability and does not need any stabilising or external cross-linking agents. Different nanoformulations with dextran sulphate–chitosan NPs have been reported, for oral delivery of insulin (Sarmento, Ribeiro, Veiga, & Ferreira, 2006), intravenous delivery of anti-angiogenesis peptides (Chen, Mohanraj, & Parkin, 2003) and controlled drug delivery for cancer (Saboktakin, Tabatabaie, Maharramov, & Ramazanov, 2010). Curcumin is a low molecular weight phytodrug having wide range of biological activities like antioxidant, anti-inflammatory, antitumourogenic, anticoagulant, antibacterial, anticarcinogenic, anti-ischemic and wound healing (Ishita, Kaushik, Uday, & Ranajit, 2004; Phan, See, Lee, & Chan, 2001; Shaikh, Ankola, Beniwal, Singh, & Ravikumar, 2009; Shukla, Khanna, Ali, Khan, & Srimal, 2008).

Curcumin has shown to be very effective against many cancer cells like breast cancer, prostate cancer, bone cancer, head and neck cancer, lung cancer and gastrointestinal cancer (Sandur et al., 2007). Though it has many good properties, it is not widely used for cancer treatment because of its poor aqueous solubility. Curcumin also faces serious problems like low gastrointestinal absorption, poor bioavailability and rapid metabolism (Pan, Huang, & Lin, 1999). In this work we have developed curcumin loaded dextran sulphate-chitosan NPs to overcome the pharmacokinetic problems and to obtain the full benefits of the drug.

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doi:10.1016/j.carbpol.2011.01.005

2. Materials and methods

2.1. Materials

Sodium salt of dextran sulphate (mol wt >500 kDa), low molecular weight chitosan (degree of deacetylation, DD >75%), was purchased from Sigma–Aldrich, curcumin from Merck India Ltd. (mol wt 365), distilled ethanol and all other chemicals used were of analytical grade. Mouse fibroblast cells (L929), human breast cancer cells (MCF-7), human prostate cancer cells (PC-3) and human osteosarcoma cell (MG63) was purchased from NCCS, Pune for cell culture experiments.

2.2. Methods

2.2.1. Preparation of dextran sulphate–chitosan NPs

0.1 wt% of chitosan solution was prepared in 1% acetic acid and 0.1% of dextran sulphate solution was made in distilled water. Dextran sulphate solution was added to chitosan solution under vigorous stirring in a volume ratio of 3:2, and dextran sulphate–chitosan NPs were formed almost instantaneously. The suspension was allowed to stir for 15 min, and then the NPs were separated from the suspension by centrifuging at 20,000 rpm for 20 min and washed twice in distilled water and re-suspended in 10 mM PBS buffer.

2.2.2. Preparation of curcumin loaded dextran sulphate–chitosan NPs

A stock solution of curcumin was prepared in distilled ethanol (2 mg/ml). Curcumin (2 mg) was loaded into the NPs in 5 wt% of polymer by taking appropriate volume from the stock solution along with the chitosan solution and the same procedure was followed as described for dextran sulphate–chitosan NP preparation. The solution was warmed (at 40 °C) in a water bath and allowed to stir for 30 min to facilitate the evaporation of ethanol. The drug-loaded suspension was centrifuged, the pellet was washed twice in water and redispersed in 10 mM PBS.

2.3. Characterization

The mean size of the prepared NPs was determined by DLS measurements and the stability of the NPs system was determined by zeta potential measurements using zeta sizer (DLS-ZP/Particle Sizer NicompTM 380 ZLS). Surface morphology of the prepared NPs was studied using SEM (JEOLJSM-6490LA) and AFM (JEOL JSPM-5200). FT-IR spectrum was also taken to study the interaction between polymers and between the drug and polymers using Perkin Elmer spectrum RXI. To see the physical state of curcumin within the NPs, thermal studies (DTA) (SII TG/DTA 6200 EXSTAR) and XRD (PANalytical X'Pert PRO X-ray diffractrometer) analysis were done.

2.4. Entrapment efficiency and loading efficiency

The entrapment efficiency of curcumin within the drug loaded dextran sulphate chitosan NPs was determined by pelletizing the sample at 20,000 rpm for 20 min. The resulting pellet was redispersed and further lyophilized. A known quantity (2 mg) of lyophilised sample was taken in 10 ml of ethanol; the solution was sonicated thoroughly using a probe sonicator (Sonics Vibra-cell, Modal-VCX 130, O/P = 130 W) at 45% of amplitude for 5 min. The resulting solution was centrifuged at 20,000 rpm for 15 min and the supernatant was collected. Amount of drug within the supernatant was quantified spectrophotometrically at an absorption maximum of 427 nm, which corresponds to the peak absorption of curcumin [using UV-1700 Pharma Spec SHIMADZU]. Entrapment efficiency [EE] was calculated based on the ratio of amount of drug present in the NPs to the amount of drug used in the loading process.

$$\mathrm{EE}\,(\%) = \frac{\text{Total amount of curcumin with in the pellet}}{\text{Initial amount of curcumin taken for loading studies}} \times 100$$

Loading efficiency (LE) of the drug-loaded system was also calculated with respect to the yield of the nanoparticles obtained after centrifugation (Anitha, Maya, et al., 2011).

$$\mathrm{LE}\,(\%) = \frac{\text{Total amount of curcumin entrapped with in the pellet}}{\text{Yield of drug loaded NPs}} \times 100$$

2.5. In vitro drug release studies

In vitro drug release profile of curcumin from drug loaded dextran sulphate–chitosan nanoparticles were done by direct dispersion method as explained in literature (Bisht et al., 2007; Anitha, Maya, et al., 2011). *In vitro* drug release studies were done for a period of one week at two different pH (7.4 and 5). A known quantity of curcumin loaded NPs were taken in 30 ml 10 mM PBS and it was divided into 30 eppendorf tubes (10 set each having 3 tubes). The tubes were then incubated in a water bath shaker at 37 °C. At definite time intervals, one set of tubes were taken out and centrifuged at 1200 rpm for 3 min to pelletize the released drug, leaving the entrapped drug within nanoparticles which stays in supernatant. The pellets were dissolved in 4 ml ethanol and amount of drug released was quantified spectrophotometerically at a wavelength of 427 nm.

2.6. Cell culture

All the cell culture experiments were done based on the previously reported method (Anitha, Maya, et al., 2011). MCF-7, MG 63 & L929 cells were maintained in MEM and PC3 in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.5% antibiotics. The cells were incubated at 37 °C with 5% CO_2. After reaching confluency, the cells were trypsinized and subcultured in the growth medium for further studies.

2.6.1. In vitro cell uptake studies using fluorescent imaging and absorption measurements

In vitro cellular uptake study of curcumin loaded dextran sulphate–chitosan NPs was done by absorption measurements. MCF-7, MG 63, L929 and PC3 cell lines were incubated in 24 well plates, with a seeding density of 50,000 cells/well. 4 mg/ml of 5 wt% curcumin loaded dextran sulphate–chitosan NPs was added to each wells and incubated. At definite time intervals, the cells were washed well with PBS and trypsinised using 5% trypsin–EDTA solution. The suspension was collected and the cells were lysed using a probe sonicator. The amount of curcumin taken up by the cells was quantified spectrophotometrically by recording the absorbance at 427 nm.

Cell imaging was also done by seeding cells on acid etched cover slips with a seeding density of 50,000 cells/cover slip and incubating at 37 °C for 24 h. The cells were then incubated with media containing curcumin loaded dextran sulphate–chitosan NPs at the same concentration that was used for cytotoxicity experiment. At definite time intervals, cover slips were taken out, washed with PBS, fixed with 2% paraformaldehyde and dried overnight in the absence of light to avoid photo bleaching of curcumin. The dried cover slips were mounted on a glass slide and imaged under fluorescent microscope (Olympus DP71 – filter number 4).

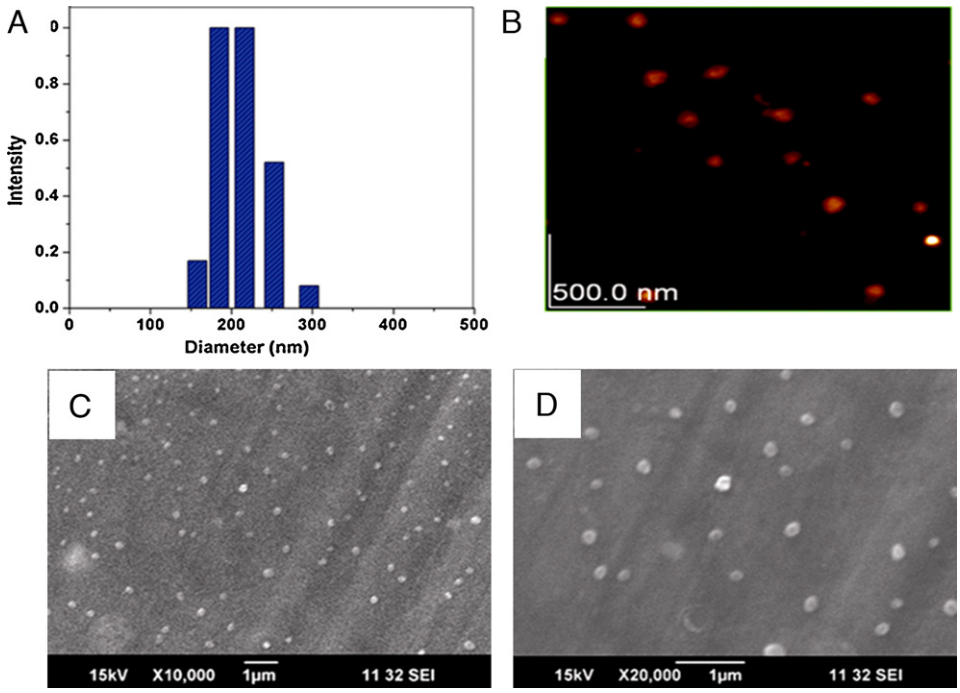

Fig. 1. (A) DLS showing the size distrubution of curcumin loaded dextran sulphate–chitosan NPs. (B) AFM image of curcumin loaded dextran sulphate–chitosan NPs. (C and D) SEM images of curcumin loaded dextran sulphate–chitosan NPs at different magnifications.

2.6.2. In vitro anticancer activity studies

2.6.2.1. MTT assay. *In vitro* cytotoxicity of bare and curcumin loaded dextran sulphate–chitosan NPs were analysed in cancer and normal cell lines by MTT assay. MCF-7, MG 63 and L929 cells were grown in MEM medium and PC3 cells were grown in DMEM medium and the study was conducted for 24 and 48 h. Cells were seeded in 96 well plates with a density of 7000 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. Then the media was removed and replenished with media containing 5 and 10% of drug loaded NPs. MTT assay was done after incubating the plates for 24 and 48 h. The plates were read for the absorbance value at 570 nm using a microplate reader (BioTek Power Wave XS). Triplicates of each sample were analysed. Anticancer activity was expressed with respect to the number of viable cells, i.e. anticancer activity is indirectly proportional to the number of viable cells and this in turn is directly proportional to optical density at 570 nm.

2.6.2.2. Apoptosis assay by flow cytometry (Annexin V-FITC/PI staining). The apoptosis assay was done according our reported method (Anitha, Maya, et al., 2011). Anticancer activity of curcumin loaded dextran sulphate–chitosan NPs was confirmed flow cytometrically via apoptosis assay in one normal (L929) and one cancer cell line (MCF-7). Phosphatidylserine (PS) exposure was detected using Annexin V-FITC/PI Vybrant apoptosis assay kit (Molecular probes, Eugene). Cells at log phase were seeded in a 24 well plate with a density of 50,000 cells/well. After attaining 90% confluency, the cells were treated with NPs with same concentrations as that of the MTT assay after diluting with media. Following exposure to curcumin loaded NPs for 24 h; cells were harvested by trypsinization and washed with ice-cold PBS for 5 min at 500 × *g* at 4 °C. The supernatant was discarded and the pellet was re-suspended in ice-cold 1× Annexin binding buffer (5×10^5–5×10^6 cells/ml), 5 μl of \xin V-FITC solution and 1 μl of PI (100 μg/ml) were added to 100 μl of the cell suspensions. The samples were mixed gently and incubated at room temperature for 15 min in the dark. After incubation, 400 μl of ice-cold 1× binding buffer was added and mixed gently, and analysed flow cytometrically. Cells in media alone, devoid of NPs, were used as negative control. Triplicate of each samples were analysed.

3. Results and discussion

3.1. Preparation of curcumin loaded dextran sulphate–chitosan NPs

Curcumin loaded dextran sulphate–chitosan NPs were formed immediately due to the co-acervation reaction between two oppositely charged polyelectrolyte polymers, i.e. dextran sulphate (negatively charged) and chitosan (positively charged). The co-acervation reaction results from the electrostatic interaction between the protonated amino groups of chitosan and sulphate groups of dextran sulphate. The size and surface charge of the prepared NPs was tunable by varying the polymer concentration and reaction conditions. These NPs have good stability and does not require any stabilisers. Curcumin loaded dextran sulphate–chitosan nanoparticles were obtained by the addition of ethanolic curcumin to chitosan solution before cross-linking with dextran sulphate.

3.2. Particle size analysis using DLS, SEM and AFM and stability studies by zeta potential measurements

The particle size data of curcumin loaded dextran sulphate–chitosan NPs by DLS showed (Fig. 1A) a size range of 180–300 nm with an average size of 220 nm. AFM (Fig. 1B) and SEM (Fig. 1C and D) images show that the average size is around 200 nm with spherical morphology. From the AFM and SEM images it is also clear that the prepared NPs have a smooth surface with spherical morphology. The NPs of 220 nm will be ideal for passive targeting of tumour as majority of solid tumours exhibit a vascular pore cut-offs between 380 and 780 nm (Zahr, Davis, & Pishko, 2006).

Stability of curcumin loaded dextran sulphate–chitosan NPs were studied by zeta potential measurements and the zeta poten-

tial value was found to be around −30 mV, indicating that the NPs have good stability. Negative surface charge of the drug loaded NPs is due to the high charge density of dextran sulphate and at neutral pH, most of the free amino group of chitosan are deprotonated. In order to achieve maximum EPR effect, the polymeric NPs should have longer circulation time and it should not trigger any unwanted immune response. It has been reported that the particles with a positive charge or hydrophobic surfaces favours plasma protein adsorption on the surface of the NPs facilitates opsonisation and are susceptible to RES clearance (Gessner et al., 2000). As the prepared nanoparticles have negative surface charge and since it is made up of hydrophilic material it might evade RES clearance as it can avoid protein adsorption.

3.3. FT-IR analysis

Fig. 2 shows the FT-IR spectra of curcumin loaded dextran sulphate–chitosan NPs (a) dextran sulphate (b), curcumin (c) and chitosan (d). In the spectrum of chitosan (d), a peak at 1648 cm^{-1} was observed and this corresponds to amide I bending vibration. The sulphate groups of dextran sulphate produces a number of vibrational bands in the region of 1200–1280 cm^{-1} as shown in the spectrum (b). The change in wave number of the peaks in these regions indicates that there is an interaction between the two groups. In the NPs spectrum, the wave number shifted from 1648 to 1636 cm^{-1} and the same condition was observed in sulphate stretching vibrations (spectrum (a)) there was a peak shift from 1271 to 1254 cm^{-1}. In the spectra of curcumin dextran sulphate–chitosan NPs (c), a peak at 1509 cm^{-1} was observed

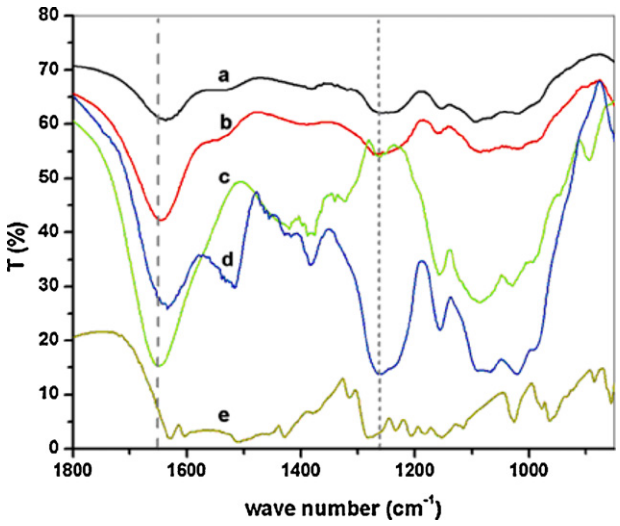

Fig. 2. FT-IR spectrum of (a) dextran sulphate–chitosan NPs, (b) dextran sulphate, (c) curcumin loaded dextran sulphate–chitosan NPs, (d) chitosan and (e) curcumin. The dashed line indicating the region of the vibrational band of amide-I of chitosan and the dotted line indicating vibrational region of sulphate ions of dextran sulphate.

which corresponds to –NH deformation and a change in the peak at 1022 cm^{-1} correspond to keto group of the curcumin. From the FT-IR data it is confirmed that the NPs were formed due to the interaction between the sulphate groups of dextran sulphate and amino groups of chitosan. There was an interaction between the

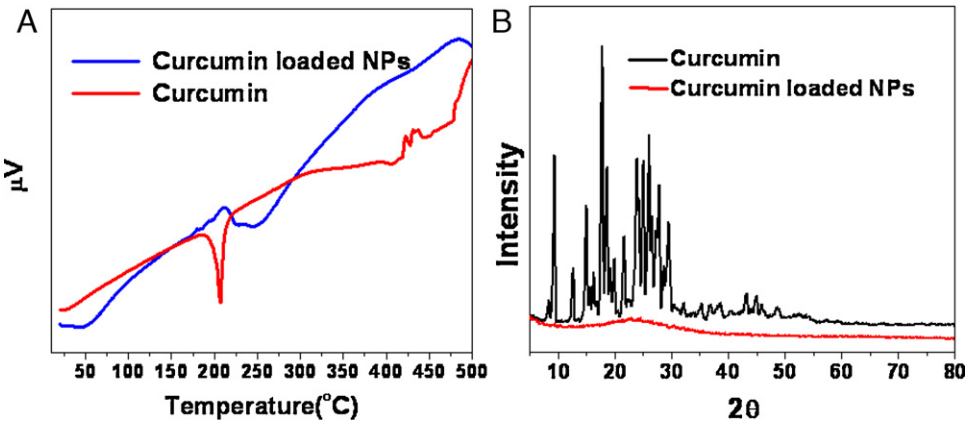

Fig. 3. (A) DTA curve of bare curcumin and curcumin loaded dextran sulphate–chitosan NPs, an endothermic peak near 220 °C of curcumin and absence of the peak at curcumin loaded NPs. (B) XRD graph showing the crystalline state of bare curcumin and amorphous state of curcumin with in the dextran sulphate–chitosan NPs.

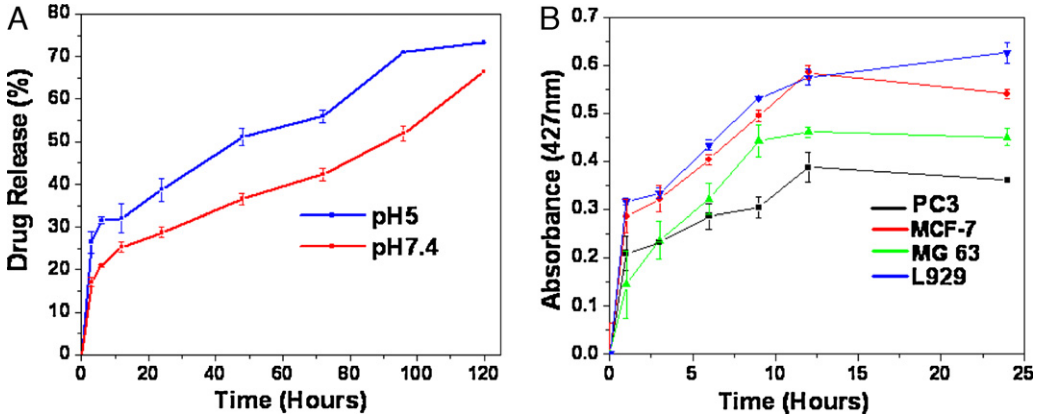

Fig. 4. (A) Drug release profile in two different pHs and (B) cellular uptake quantification by spectrophotometric method in L929, MCF-7, PC3 and MG 63 cells.

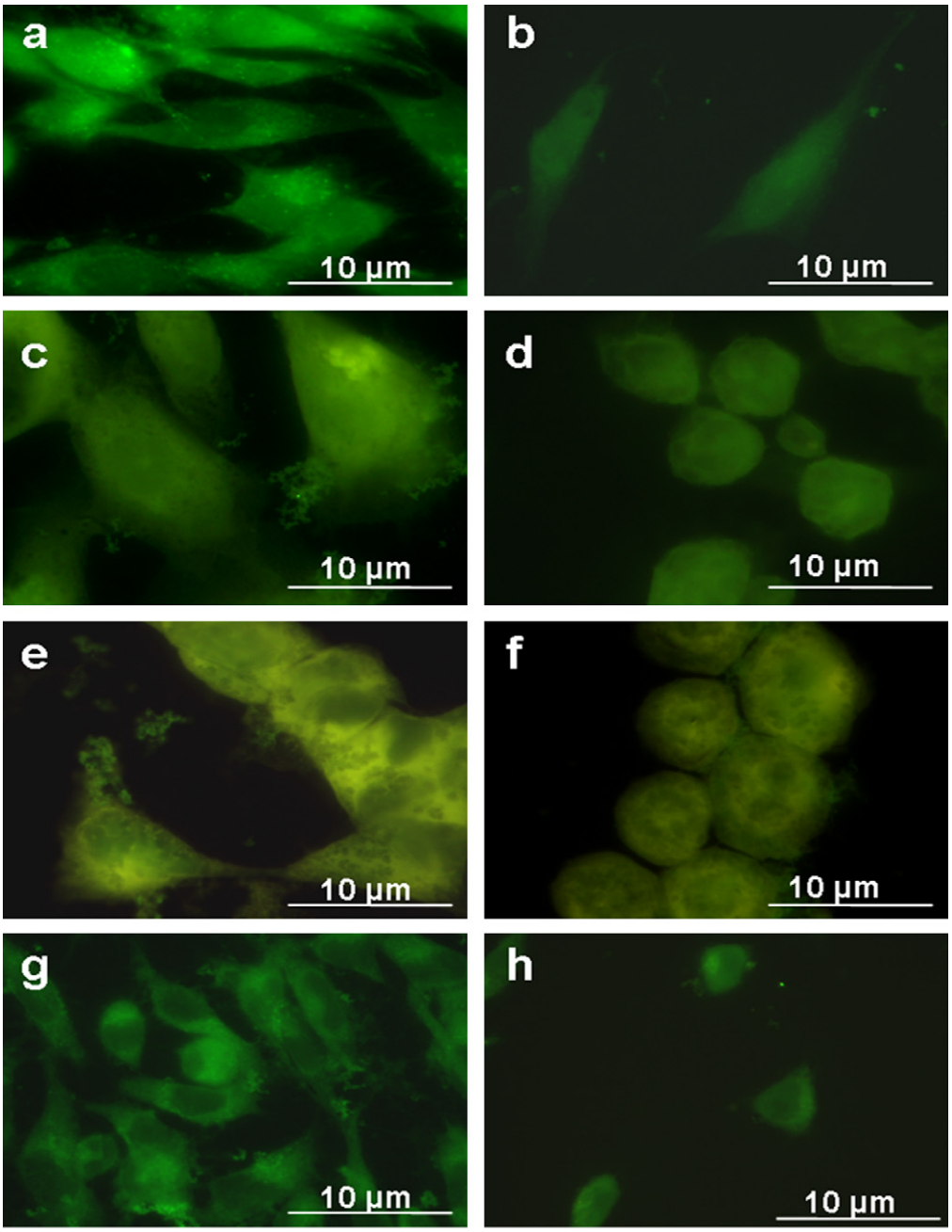

Fig. 5. Fluorescent images of L929 (a & b), PC3 (c & d), MCF-7 (e & f) and MG 63(g & h) taken at 1st and 24th h of incubation respectively.

keto group of curcumin and amine group of chitosan that can also cause drug loading. The interaction can help in reducing the release rate of curcumin thereby aiding controlled release.

3.4. Physical nature of curcumin with in the NPs system

As curcumin is a highly hydrophobic drug, it tends to form crystals when curcumin solution is added to an aqueous solution. If the nano sized crystals are formed inside the matrix of the NPs, then the drug elution from the NPs will be hindered and the release profile will be irregular. In the DTA (Fig. 3A) analysis, curcumin showed a strong endothermic peak at temperature around 220 °C, due to crystalline nature of curcumin, whereas the NPs displayed no peak in this region. Also in XRD data of curcumin (Fig. 3B) a number of peaks were observed in the 2θ range of 10–30° implying its crystalline nature, but in the nanoformulation of curcumin, there were no such crystalline peaks. This again confirms the amorphous or disordered-crystalline phase of curcumin in the NPs.

3.5. Entrapment efficiency and in vitro drug release studies

The entrapment efficiency and loading efficiency of 5 wt% curcumin loaded NPs system was found to be ≈74% and 4.4%, at higher drug concentrations, the entrapment efficiency and loading efficiency was found to be decreasing.

In vitro drug release studies were done via the direct dispersion method at pH 5 and 7.4 and the release pattern is shown in Fig. 4A. The drug release pattern showed a burst release in the first 3 h followed by a controlled release of curcumin over a period of one week and about 70% of drug was released during this time. Curcumin that is adsorbed on to the NPs surface and drug entrapped near the surface might be the reason for initial burst release, as the dissolution

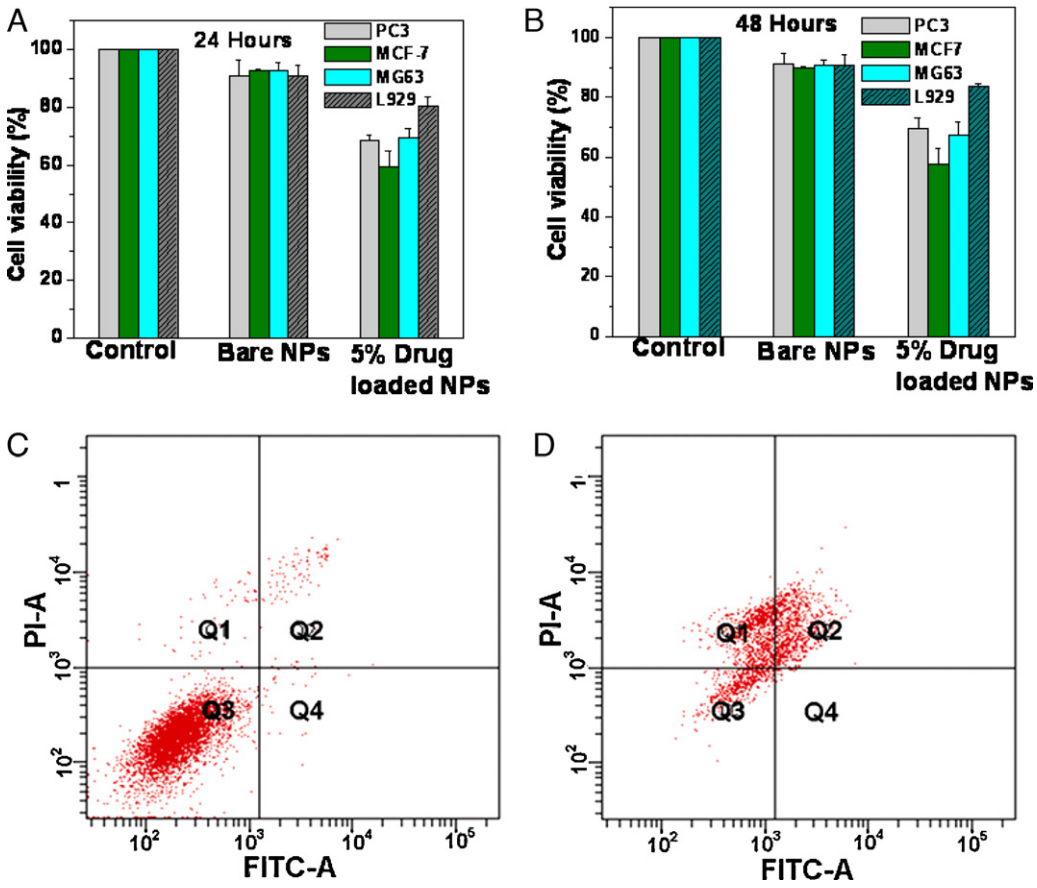

Fig. 6. (A and B) Cytotoxicity studies by MTT assay (24 and 48 h) for dextran sulphate-chitosan NPs and curcumin loaded dextran sulphate-chitosan NPs.(C and D) FACS analysis showing less apoptosis in L929 cells and more apoptosis in MCF-7 cells after 24 h incubation.

rate of the polymer near the surface is high, the amount of drug released will be also high. The release was faster in acidic pH than in neutral, as in acidic environment the polymer matrix swells due to protonation of amine group of chitosan, thereby facilitating the faster drug elution (Hobbs et al., 1998; Jayakumar, Reis, & Mano, 2007).

Also, most of the tumours have poor blood vessel architecture, and as a result the metabolic end products accumulate in the tumour microenvironment resulting in acidic pH. Hence, the NPs accumulated in the tumour site would release its content more quickly as the NPs swell due to protonation of chitosan.

3.6. *In vitro cell uptake study*

In vitro cell uptake studies of curcumin loaded dextran sulphate–chitosan NPs were done by fluorescent imaging and quantification was done spectrophotometrically. The curcumin uptake quantification by spectrophotometric method was shown in Fig. 4B. Cellular uptake of curcumin by the cells in log phase was found to increase with respect to time for the first 15 h and in later part, the rate of uptake slows down as curcumin starts to inhibit the cell proliferation or cause cell death via apoptosis.

The *in vitro* cell uptake studies via fluorescent imaging were done at 1st and 24th hour and the images are shown in Fig. 5. There was a little difference observed in the uptake of nanoparticles by different cells. All cancer cell lines at the 24th h of incubation with NPs showed spherical morphology than the cell incubated for 1 h, with the L929 cell lines being affected the least. Uptake increases with incubation time, with no significant differences between normal and cancer cells. However, curcumin showed selective toxicity towards cancer cells. This may be due to the fact that curcumin targets many signalling molecules, which cancer cells highly rely on (Walters, Muff, Langsam, Born, & Fuchs, 2008).

3.7. *In vitro cytotoxicity by MTT assay and apoptosis assay by FACS analysis*

The anticancer activity of curcumin loaded dextran sulphate–chitosan NPs was analysed by MTT assay (Fig. 6A and B) and FACS (Fig. 6C and D). MTT results (Fig. 6A and B) showed that the drug loaded NPs were toxic to cancer cells, whereas bare NPs showed very little toxicity for both cancer and normal cells. Cell viability of 5 wt% curcumin loaded NPs were higher in MCF-7 (59.5 and 57.9% respectively for 24 and 48 h incubation), for MG 63 (76 and 69.6%), for PC3 (72.6 and 68.67%) and for L929 cells (82.3 and 80.5%).

4. Conclusions

Curcumin loaded dextran sulphate–chitosan NPs were prepared by simple co-acervation method. Studies related to the entrapment efficiency; *in vitro* drug release, cell uptake and cytotoxicity of the prepared NPs were conducted. The prepared NPs have a spherical morphology with negative zeta potential and good colloidal stability. In this study 70% of curcumin was released after 120 h and the drug release was better when compared to our previous system of curcumin loaded *O*-carboxymethyl chitosan NPs (Anitha, Maya, et al., 2011). These results suggest that dextran sulphate–chitosan NPs could be used as an ideal carrier to deliver hydrophobic drugs like curcumin in cancer drug delivery.

Acknowledgements

The authors are thankful to Department of Biotechnology (DBT), Govt. of India, for their financial support for this work under the Nanoscience and Nanotechnology Initiative program (Ref. No. BT/PR10882/NNT/28/142/2008). This work was also partially supported by Nanomission, Department of Science and Technology, India under the Nanoinitiative Program monitored by Prof. C.N.R. Rao. One of the authors A. Anitha is thankful to Council of Scientific and Industrial Research (CSIR), India for providing Senior Research Fellowship (SRF award-Ref. No. 9/963 (0005) 2K10378-EMR-I) for carrying out her research work. The authors are also thankful to Koyochemicals Co. Ltd., Japan for providing Chitosan. The authors are also thankful to Mr. Sajin P. Ravi, Mr. Girish C.M., Mr. Sarath, Mrs. Sreerekha P.R. and Mr. Sudheesh Kumar P.T. for their helps in SEM, AFM, FACS and TG/DTA studies.

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