Carbohydrate Polymers 87 (2012) 2352–2356

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Short communication

Doxorubicin-loaded pH-responsive chitin nanogels for drug delivery to cancer cells

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ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form 12 October 2011
Accepted 15 October 2011
Available online 20 October 2011

Keywords:
Doxorubicin loaded chitin nanogels
Cellular uptake
Swelling
pH responsivity
Cancer drug delivery

ABSTRACT

This work deals with preparation of doxorubicin loaded chitin nanogels and were characterized by SEM, DLS and FTIR for cancer drug delivery. The *in vitro* cytotoxicity studies of 130–160 nm sized doxorubicin loaded chitin nanogels were studied using MTT assay on L929, PC3, MCF-7, A549 and HEPG2 confirmed that relatively higher toxicity on cancer cells comparing to normal L929 cells. The internalization studies showed a significant uptake of doxorubicin loaded chitin nanogels in all the tested cell lines. All the above results indicated that doxorubicin loaded chitin nanogels can be used for prostate, breast, lung and liver cancer.

1. Introduction

Chitin, composed of (1–4) linked units of N-acetyl-β-D-glucosamine (some of which deacetylated), exists in the form of nanostructures in living organisms (Muzzarelli, 2011a). It is being used in the fabrication of a variety of medical devices, and regenerative medical components, thanks to its high crystallinity, biochemical significance and biocompatibility (Yimin, Tsuguyuki, & Akira, 2008). The inherent and unique properties of large and active surface area are being explored in the fields of engineering, technology and medicine (Muzzarelli, 2011b). Nanohydrogels (nanogels) are cross-linked nanoparticles made of flexible hydrophilic polymers. They swell in water and allow spontaneous loading of drugs in aqueous media (Raemdonck, Joseph, & De Smedt, 2009). They can be thus made responsive to stimuli and to changes in the environment, such as pH and temperature. Different approaches have been running in the current era to cure the cancer through encapsulating different anticancer drugs in polymeric nanomaterials. The advanced cancer treatments in terms of developing smart carrier molecules and delivering them at specific site are required treating metastatic cancers. Thus the main objective of our work is to study the efficiency of these doxorubicin loaded chitin nanogels in the controlled delivery of the doxorubicin and to analyze their cytotoxicity against the cancer cell lines *in vitro*.

2. Materials and methods

2.1. Materials

Chitin (degree of acetylation – 72.4% and 150 kDa) from Koyo chemical Co., Ltd., Japan, $CaCl_2$ and methanol from Qualigens, India. Doxorubicin from AIMS, Kochi. Cell lines from National Center for Cell Sciences, Pune, India.

2.2. Preparation of chitin nanogels and doxorubicin loaded chitin nanogels

Chitin nanogels were prepared according to our previous method (Sanoj Rejinold, Amrita Nair, et al., 2012 Sanoj Rejinold, Chennazhi, Tamura, Nair, & Jayakumar, 2011). To prepare doxorubicin loaded chitin nanogels, where the weight of doxorubicin is 10% of the weight of chitin nanogels, about 125 μL (250 μg) is added to 1 ml (2.5 mg/ml) of chitin nanogels. This is then kept under stirring for 5 h to allow for proper loading of doxorubicin into the chitin nanogels, then centrifuged to remove excess drug. The pellet is then resuspended in water to be used for further studies. The solution was pink due to the doxorubicin entrapped within the chitin nanogels.

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doi:10.1016/j.carbpol.2011.10.040

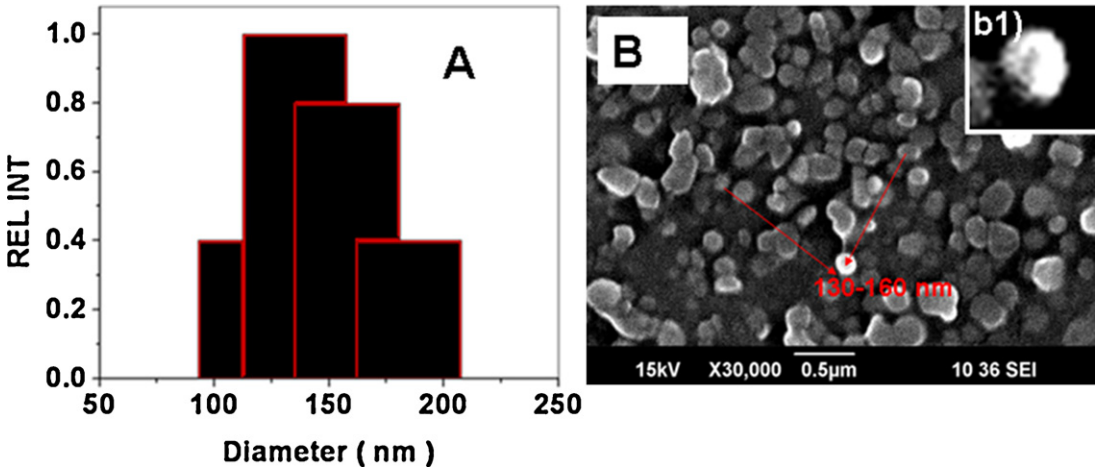

Fig. 1. Particle size analysis of doxorubicin loaded chitin nanogels by (A) DLS and (B) SEM; inset (b1) showing the magnified SEM image for the doxorubicin–chitin nanogels.

2.3. Loading efficiency of chitin nanogels and in vitro drug release studies of doxorubicin loaded chitin nanogels

The loading efficiency and *in vitro* drug release study was carried out by the following method (Sanoj Rejinold et al., 2011). Pellets of the prepared doxorubicin loaded chitin nanogels were immersed in PBS buffer at pH 7.4 and 4.5. The samples were maintained at 37 °C and placed in an incubated shaker at 120 rpm. At predetermined intervals, aliquots of 100 μL were withdrawn and replaced with an equal volume of fresh buffer. These samples were aliquoted into labeled 96 well plate. The concentration of the drug was monitored with a plate reader at the wavelength of 481 nm. The percentage drug release was determined by the following equation:

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

where $c(0)$ and $c(t)$ represent the amount of drug loaded and amount of drug released at a time t, respectively. All experiments were done in triplicate.

2.4. Cell culture protocols

L929 (mouse fibroblast cell line, NCCS Pune), MCF-7 (human breast cancer cell line), A549 (adenocarcinomic human alveolar basal epithelial cells); and HEPG2 (human liver cancer cells) were used for this study. The toxicity of doxorubicin loaded chitin nanogels has been analyzed for five different concentrations, viz. (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) using the existing protocols for a period of 48 h. For uptake studies, acid etched cover slips kept in 24 well plates were loaded with L929, PC3, MCF7, A549 and HEPG2 cells and incubated for 24 h. Further studies were done in accordance with our previous protocols (Sanoj Rejinold et al., 2011).

2.5. Characterizations of doxorubicin loaded chitin nanogels

The FT-IR spectra were taken using Perkin Elmer Spectrum RX1 Fourier transform infrared spectrophotometer using KBr tablets. The mean size and size distribution were determined by dynamic light scattering (DLS-ZP/Particle Sizer Nicomp™ 380 ZLS) measurements. Size of the particles was done using SEM (JEOLJSM-6490LA).

2.6. Statistics

Statistical analysis of the data was performed via one-way analysis of variance (ANOVA) using origin software; a value of $p < 0.05$ was considered significant ($n = 3$).

3. Results and discussion

3.1. Preparation and characterization of doxorubicin loaded chitin nanogels

A required volume of doxorubicin was aspirated to the prepared chitin nanogels and kept for 5 h stirring. The longer the incubation is enhancing the higher loading of the drug. However the size of the nanogel was affected as before because of the surface adsorption of the drug.

The size distribution for the prepared nanogels was found to be 50–80 nm (Sanoj Rejinold et al., 2012) and 130–160 nm for the doxorubicin loaded chitin nanogels (Fig. 1A). The SEM images also confirmed the same size range for the doxorubicin loaded chitin nanogels (Fig. 1B), which is having a spherical morphology (Fig. 1Bb1).

Fig. 2a–d shows the FT-IR spectra of doxorubicin loaded chitin nanogels, bare doxorubicin, chitin nanogels and chitin control. The characteristic peaks of doxorubicin showed at 2932 (C–H stretching vibrations), 878 and 805 cm^{-1} (N–H wagging), 1260 (stretching of alcoholic O–H groups), 1410 (C–C stretching), 794 (NH_2 and N–H wagging) (Kayal & Ramanujan, 2010). On comparing the chitin nanogels and the doxorubicin loaded chitin nanogels, the peak at 1070 cm^{-1} (C–C stretching) is the same in both chitin nanogels and doxorubicin loaded chitin nanogels. As seen for doxorubicin loaded chitin nanogels, peaks corresponding to the stretching of the hydroxyl groups (3480–3450 cm^{-1}) are more broadly distributed when compared to the bare nanogel. This could be due to the interaction between the nanogels. On comparing the doxorubicin and the doxorubicin loaded chitin nanogels, it was observed that the peaks at 878 and 805 cm^{-1} were present in doxorubicin loaded chitin nanogels; but were diminished slightly. The doxorubicin peak at 1260 cm^{-1} was seen in doxorubicin loaded chitin nanogels confirming the presence of doxorubicin in the chitin nanogels. There was also a shift from 794 to 798 cm^{-1}, confirming the presence of intermolecular hydrogen bonding between the doxorubicin and doxorubicin loaded chitin nanogels (Kayal & Ramanujan, 2010).

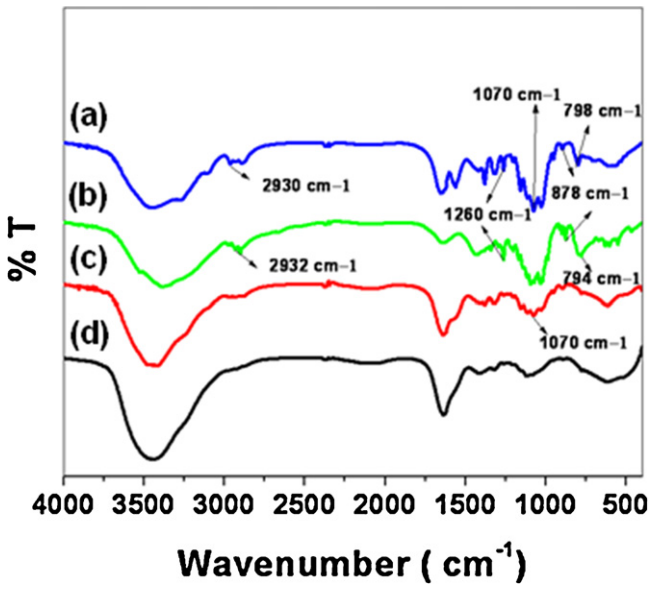

Fig. 2. FTIR spectra of (a) of doxorubicin loaded chitin nanogels; (b) bare doxorubicin; (c) chitin nanogels; (d) chitin control.

3.2. Loading efficiency and in vitro drug release profiles

The increase in incubation time was the only factor that affected the loading efficiency, and the loading efficiency increased to about 26% within 5 h. The release studies showed that doxorubicin release was more in acidic pH compared to neutral pH, (Fig. 3A). First hour 32% release was somewhat similar in acidic and neutral pH. 60% of the drug was released within 24 h under acidic, whereas only about 40% is released in the neutral environment. The release could be due to swelling in acidic pH as a combination of simple diffusion, nanogel degradation, change in pH and the displacement by counterions present in the environment (Sanoj Rejinold et al., 2011, 2012). For a polyelectrolyte like the chitin nanogel, fixed charges form on the nanogel network when the nanogel is exposed to an environment of appropriate pH, because of the ionization of the pendant groups. This in turn causes electrostatic repulsion in the nanogels network thereby resulting in the enlargement of the pores and allowing excess solvent influx.

3.3. Cytotoxicity and cell uptake studies

Cytotoxicity studies reveal that the doxorubicin loaded chitin nanogels showed a significant toxicity towards the tested cell lines (Fig. 3B), compared to normal L929 cells. doxorubicin loaded chitin nanogels showed a significant uptake in all cells tested (Fig. 4). The exact mechanism of action of doxorubicin is complex and unclear,

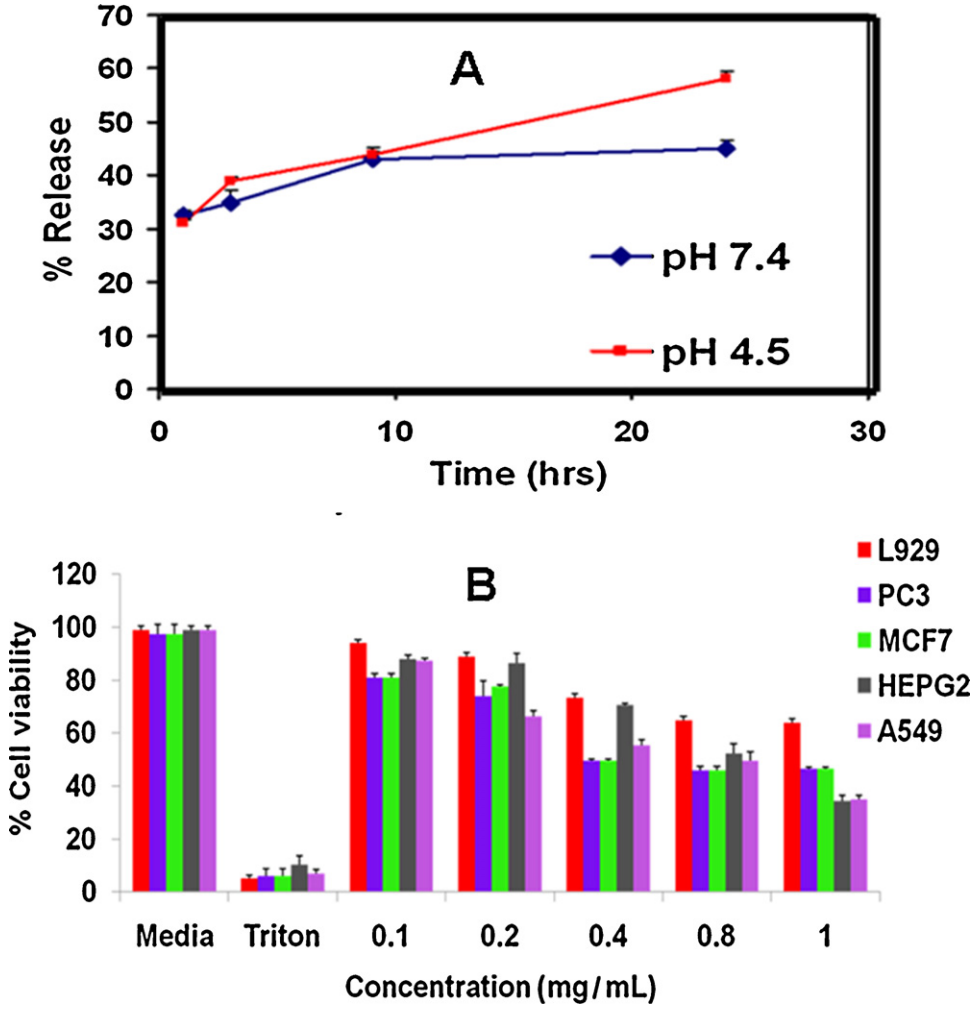

Fig. 3. (A) Release profile of doxorubicin from doxorubicin loaded chitin nanogels at pH 7.4 and pH 4.5 and (B) cytotoxicity studies of doxorubicin loaded chitin nanogels on L929, PC3, MCF7, HEPG2 and A549 cells.

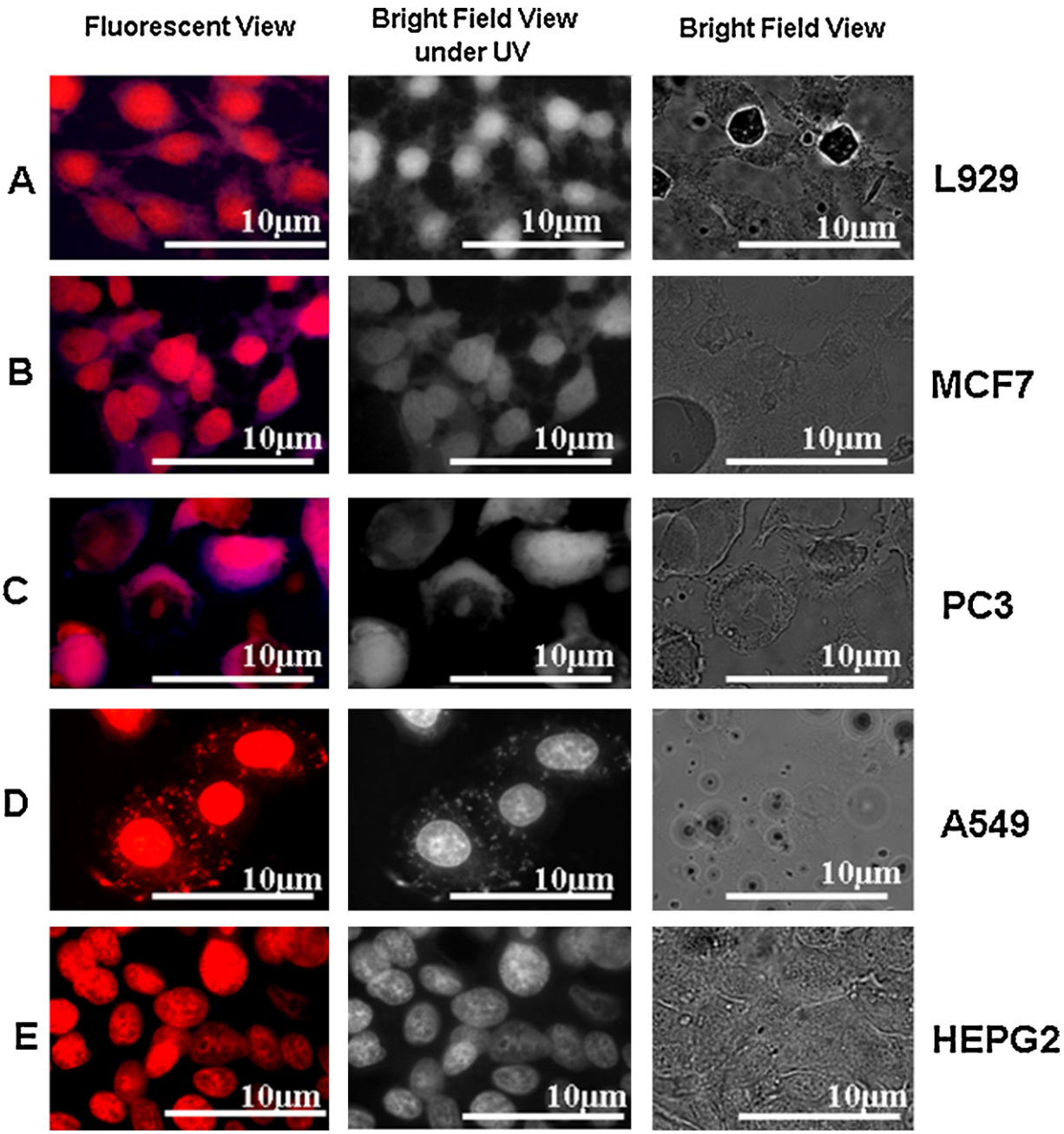

Fig. 4. Fluorescent microscopy images of doxorubicin loaded chitin nanogels taken up by (A) L929, (B) MCF7, (C) PC3, (D) A549 and (E) HEPG2 cells after 4 h of incubation period.

though it is thought to interact with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication (Kate, Suzanne, Ted, Paul, & Don, 2008).

4. Conclusion

The biodegradable and biocompatible doxorubicin loaded chitin nanogels (130–160 nm) was prepared and characterized for cancer drug delivery applications. The doxorubicin loaded chitin nanogels showed pH sensitive controlled release of doxorubicin. Cytotoxicity studies showed that doxorubicin loaded chitin nanogels are toxic to all tested cancer cells sparing normal L929 cells. The cell uptake studies reveal that doxorubicin loaded chitin nanogels could enter and illuminate the cells for imaging them with therapy. These studies indicated that the doxorubicin loaded chitin nanogels could be a better alternative for cancer therapeutic agent.

Acknowledgments

The Department of Biotechnology, Government of India supported this work, under a center grant of the Nanoscience and Nanotechnology Initiative program (Ref. No. BT/PR10850/NNT/28/127/2008). This work is also partly supported by Department of Science and Technology under the Nanoinitiative programme. The authors, N. Sanoj Rejinold and S.

Maya acknowledge Council of Scientific and Industrial Research (CSIR) for the financial support through Senior Research Fellowships (N. Sanoj Rejinold; SRF; Award No. 9/963 (0017)2K11-EMR-I) and (S. Maya; Award No. 9/963 (00172)2K11-EMR-I), respectively.

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