International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Antioxidant studies of chitosan nanoparticles containing naringenin and their cytotoxicity effects in lung cancer cells

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ARTICLE INFO

Article history:
Received 21 December 2014
Received in revised form 12 March 2015
Accepted 12 March 2015
Available online xxx

Keywords:
Chitosan
Nanoparticles
Naringenin
Lung cancer cells

ABSTRACT

Chitosan based nano carrier systems have been widely explored owing to its reliability and simpler synthesis route. In the current study, chitosan (CS) encapsulated naringenin (NAR) nanoparticles (CS-NPs/NAR) were synthesized by ionic gelation method mediated by tripolyphosphate (TPP) as a cross-linker and characterized by DLS, SEM, Zeta potential, FT-IR and EDS analyses. The encapsulation efficiency of CS-NPs/NAR was determined by Folin–Ciocalteau (FC) and high performance liquid chromatography (HPLC) techniques. The native CS-NPs were found to be sized at 53.2 nm, while an increase in the size to 407.47 nm was observed upon loading with NAR. The encapsulation efficiency of CS-NPs/NAR was identified to be ~70% by FC method and ~80% by HPLC method, respectively. The release of NAR from CS-NPs/NAR in simulated gastric fluid was found to be ~15% and remaining 85% of NAR was entrapped in CS-NPs/NAR. Furthermore, the free radical scavenging ability of CS-NPs/NAR was studied by Nitrate scavenging, 2, 2-diphenyl-2-picryl hydrazyl hydrate and hydroxyl radical scavenging assays. The free radical scavenging activity was significantly higher in CS-NPs/NAR. MTT based cytotoxic analysis also depicted the non-toxic nature of CS-NPs/NAR towards normal fibroblast 3T3 cells, while cytotoxic effects were noticed against A549 lung cancer cells. Hence, the current investigations showed the superiority of chitosan encapsulated NAR over free NAR and suggested an efficient system for delivering NAR with antioxidant and anticancer activities.

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1. Introduction

Chitosan (CS) is a naturally occurring polysaccharide obtained by partial *N*-deacetylation of chitin [1]. It is used as a nanocarrier because of its unique properties such as biodegradability, biocompatibility, hydrophilicity and non-toxicity and is also inexpensive [2–4]. Chitosan (CS) also has antimicrobial activity and is more abundant on earth after cellulose [5]. Chitosan (CS) has been found to have unique properties such as capability to interact with various epithelia and also its mucoadhesion potential. Also the CS-NPs protects the drug loaded in it from external factors such as pH, enzymes etc [6]. Nano encapsulations of polymers are effective drug delivery carriers due to more surface area to adsorb, bind and carry the drugs [7]. Nano encapsulation of drug is mainly done as it protects the drug from metabolic activity, promotes controlled and sustained release of drug and it also increases the bioavailability of the drug [8]. Because of these unique properties, CS is used for the encapsulation of the flavonoid naringenin. Chitosan nanoparticles (CS-NPs) have been previously used as drug carriers/delivery systems [9–11]. Tri polyphosphate (TPP) can be used as a cross linker and it increases ionic strength of the solution by promoting ionic interaction of amino groups of chitosan with anionic groups of them [12,13]. CS-NPs produced by ionic cross linking with TPP showed increased drug loading efficiency and also prolonged drug release period [14,15]

Oxidative stress is a condition in which cellular antioxidant defence mechanism becomes insufficient to inactivate reactive oxygen species (ROS) or production of excess ROS [16]. Oxidative stress leads to severe damage to DNA, lipids and sugars [17]. Flavonoids are the plant secondary metabolites that have the ability to provide protection against the ROS which are generated as a result of oxidative stress [18]. ROS are the potential carcinogens as they facilitate mutagenesis, cancer promotion and progression [19]. It was shown that flavonoids have anti carcinogenic effect by interfering with the progression of cancer cells by modifying the process of cell proliferation, apoptosis and metastasis [20]. Naringenin (NAR) is a citrus flavonone, an effective flavonoid and is a potential anti-inflammatory agent [21]. Naringenin is soluble in ethanol and water mixture [22] and the half life of NAR has been

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http://dx.doi.org/10.1016/j.ijbiomac.2015.03.045
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reported as 2.3 h [23]. CYP3A4, an isozyme of cytochrome P450 is responsible for the rapid oxidation of drug [24] and is also involved in the activation of carcinogen [25]. Thus, flavonoids are required for the inhibition of CYP3A4 for improved drug bioavailability [26]. The mechanism behind the inhibition of CYP3A4 enzyme by the flavonoid naringenin are post transcriptional [27]. NA|R is a natural polyphenolic compound which has been revealed to have significant antitumour effects with low toxicity [28]. It has the highest potential to scavenge ROS. Hence, the objective of this study was to prepare and characterize CS-NPs encapsulated with NAR and to determine the effect of NAR released from CS-NPs/NAR for the free radical scavenging in in vitro system and cytotoxic activities in lung cancer cells (A549).

2. Materials and methods

2.1. Materials

Chitosan (CS), naringenin (NAR), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), Folin's phenol reagent, DPPH, sodium tri polyphosphate (TPP), acetonitrile were purchased from Sigma Aldrich, USA. A549 lung cells and 3T3 cells were purchased from National Centre for Cell Sciences (NCCS), Pune, India. All other chemicals used were of analytical grade.

2.2. Preparation of CS-NPs encapsulated with NAR

CS-NPs were prepared by the ionic gelation technique as described previously [29]. Briefly, CS to TPP ratios of 2:1 and 5:1 (w/w) were prepared in three different concentrations of NAR (0.1, 0.3 and 0.5 mg/ml) and stirred to get complete dissolution at pH 4.8. TPP was added in drops to the respective concentration of CS solution and stirred for 1 h and stirring was continued for another 1 h after the addition of 1 ml of trehalose (1.7 mg/ml). This mixture was then centrifuged at 10,000 rpm for 30 min; the pellet (CS-NPs/NAR) was lyophilized and used for further studies.

2.3. Characterisation

2.3.1. Zeta potential

The Zeta potential (ζ) of CS-NPs/NAR was estimated by measuring the electrophoretic mobility (U_E) using a folded capillary cell with a Zetasizer Nano Series instrument (Horiba Nano partica SZ-100, Japan). One millilitre of diluted CS-NPs/NAR particles was used for analysis. All measurements were made in triplicate.

2.3.2. Dynamic light scattering (DLS)

Particle size of CS-NPs and CS-NPs/NAR for both CS:TPP mass ratios (2:1 and 5:1) were determined using a Zetasizer Nano Series instrument (Horiba Nano partica SZ-100, Japan). The particles were resuspended in 10 ml of ddH_2O and were stirred overnight at 250 rpm. 0.5 ml of this solution was diluted with 9.5 ml of ddH_2O and was sonicated for 10 min to break the aggregated particles and the size of the particle was determined. All the samples were measured with $n = 5$.

2.3.3. Scanning electron microscope (SEM)

The sizes of CS-NPs and NAR loaded CS-NPs (CS-NPs/NAR) were found using Scanning electron microscope (FEI Quanta 200, Hillsboro, USA). The particle suspension was diluted to one-fifth with ultrapure water. A drop of the diluted particle suspension was deposited on a polished aluminium holder, was coated with thin layer of gold film. Then it was dried in vacuum and the morphology of the CS-NPs and CS-NPs/NAR were determined.

2.3.4. Fourier transformed infrared spectroscopy (FT-IR)

The characteristic bonds and the functional groups present in CS-NPs, NAR and CS-NPs/NAR were analysed in infrared spectrophotometer (Spectrum RXI, PerkinElmer, California, USA). The samples were mixed with potassium bromide to form a fine powder and then it was compressed to a thin pellet which was later analysed within the range. The infrared absorption spectra of NAR, CS-NPs and CS-NPs/NAR were examined with a wave number ranging from 500 cm^{-1} to 4000 cm^{-1} and a resolution of 0.2 cm^{-1} at room temperature. The resulting FT-IR spectral pattern was then analysed and matched with the corresponding characteristic bond and functional groups.

2.3.5. Energy dispersive spectroscopy (EDS)

The elemental compositions of the CS-NPs and CS-NPs/NAR were analysed using Energy dispersive spectroscopy (Bruker, Quantax, Ewing, NJ. USA). The particles were placed in an epoxy resin and analysed under the electron beam. Elements with atomic number greater than 5 (boron) can be analyzed by this method. Opaque samples were embedded in epoxy-resin blocks and a conducting surface coat was applied to provide a path for an incident electron flow. Vacuum-evaporated carbon was used as coating material.

2.3.6. UV analysis

The UV analysis for CS, NAR and CS-NPs/NAR were performed on a UV–vis spectrophotometer (Ultrospec 2100 pro, England).

2.4. Entrapment efficiency assessment

The efficiency of entrapment of NAR within the CS-NPs was determined by measuring the concentration of NAR in supernatant compared to their original concentrations used in preparation of CS-NPs/NAR. The entrapment efficiency was found by determining the concentration of NAR in the supernatant by using the high performance liquid chromatography (HPLC) with photodiode array detection (PDA) and also by the Folin–Ciocalteau (FC) method as follows.

2.4.1. High pressure liquid chromatography-photodiode array (HPLC-PDA)

The detection of NAR was carried out using an Agilent 1260 series HPLC with PDA detector G1315D, auto sampler G1329B with pump G1311C using C18 column (ZORBAX Eclipse plus C18 analytical column 4.6 × 250 mm with 5 micron particle size). 5 Different concentrations of NAR of 0.25 mg/ml to 1.25 mg/ml were run as standards. 20 μl of the sample was injected and was run in a flow rate of 0.8 ml/min with the mobile phase. The mobile phase consisted of Milli Q water (solvent A) and acetonitrile (solvent B). The gradient system was used initially with 100% of solvent A followed by linear gradient to 55% of solvent B by 15th minute. NAR was detected at PDA by monitoring at 240, 280, 360 and 520 nm. All samples were syringe filtered before analysis and were run in triplicate.

2.4.2. Folin–Ciocalteau (FC) method

The amount of NAR was determined by FC method with modifications of Singleton et al. [30]. In this method, the poly phospho tungstates–molybdates was reduced by a phenol compound to a blue coloured chromogen which was measured at 765 nm. 1 ml of supernatant of the sample was added to 5.0 ml of 1:10 (v/v) of FC phenol reagent and was incubated for 5 min at room temperature. Then, 4 ml of 15% of sodium carbonate was added to each tube and was again incubated for 2 h and measured at 765 nm using UV–vis spectrophotometer (Ultrospec 2100 pro, England). NAR standards

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Please cite this article in press as: S.P. Kumar, et al., Int. J. Biol. Macromol. (2015), http://dx.doi.org/10.1016/j.ijbiomac.2015.03.045

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