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Fabrication, characterization and bioevaluation of silibinin loaded chitosan nanoparticles

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ABSTRACT

Silibinin is reported to possess multiple biological activities. However, its hydrophobic nature limits its bioavailability compromising *in vivo* biological activities. Nanoparticles-based delivery of such molecules has emerged as new technique to resolve these issues. Bio-degradable, compatible and adhesive nature of chitosan has recently attracted its suitability as a carrier for biologically active molecules. This study presents fabrication and characterization of chitosan-tripolyphosphate based encapsulation of silibinin. Various preparations of silibinin encapsulated chitosan-tripolyphosphate nanoparticles were studied for particle size, morphology, zeta-potential, and encapsulation efficiencies. Preparations were also evaluated for cytotoxic activities *in vitro*. The optimized silibinin loaded chitosan nanoparticles were of 263.7 ± 4.1 nm in particle size with zeta potential 37.4 ± 1.57 mV. Nanoparticles showed high silibinin encapsulation efficiencies ($82.94 \pm 1.82\%$). No chemical interactions between silibinin and chitosan were observed in FTIR analysis. Powder X-ray diffraction analysis revealed transformed physical state of silibinin after encapsulation. Surface morphology and thermal behaviour were determined using TEM and DSC analysis. Encapsulated silibinin displayed increased dissolution and better cytotoxicity against human prostate cancer cells (DU145) than silibinin alone.

1. Introduction

Silibinin (SLB) is a polyphenolic flavonoligan and is a major biologically active component in silymarin which is derived from seeds of the milk thistle plant, *Silybum marianum* [1,2]. It is widely used for treatment of various acute and chronic liver toxicities, inflammation, fibrosis, and oxidative stress [3–5]. Various studies indicate that SLB is also active against different carcinomas like breast [6], lung [7], colon [8,9], brain [10] and prostate cancer [11]. SLB has shown anti proliferation activities through cell cycle regulation, apoptosis induction, chemosensitization, growth inhibition, anti-inflammation, inhibition of angiogenesis, reversal of multi-drug resistance and inhibition of invasion and metastasis [12–14]. Bulky multi-ring structure and poor oral bioavailability (23–47%) of SLBs leads to its low aqueous solubility and hence its clinical role is limited [3,15,16].

Nano-drug delivery systems such as nanoparticles, nanomicelles, nanosuspension and nanoemulsion have great potential to deliver the hydrophobic drugs in improved manner [17,18]. Many approaches have been employed to improve the efficacy and bioavailability of SLB, such as silybin-phospholipid complex [19], dendrimers [15], nanosuspension [20], silybin nano-structured lipid carriers [6,3], silibinin hydrogel [16] and self-emulsifying drug delivery system [21].

Biodegradable polymeric nanoparticles have attracted much attention to overcome drug associated problems [22]. Chitosan is a naturally occurring cationic polysaccharide derived by deacetylation of chitin. Chitosan based formulations have been used for the delivery of pharmaceutically active ingredients, nucleic acids [23], protein therapeutics and antigens [24]. Due to the biocompatible, biodegradable, non-toxic, non-immunogenic and noncarcinogenic properties, chitosan is widely used for the preparation of nanoparticles [25,26]. Because of the mucoadhesive

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and permeation-enhancing properties of chitosan, its nanoparticles are very promising carriers for oral drug delivery. Chitosan-TPP nanoparticles have been previously used to encapsulate various natural compounds like rutin [27], quercetin [28], tea catechins [29], curcumin [30] and ascorbic acid [31]. Chitosan nanoparticles are generally prepared by using cross-linking agents like glutaraldehyde, tripolyphosphate and polyaspartic acid [32,33].

The objective of the present study was to fabricate the silibinin encapsulated chitosan-TPP nanoparticles for oral delivery and to evaluate the potential of encapsulated SLB for anticancer activity. The nanoparticles were characterized for various physicochemical properties using dynamic light scattering (DLS), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and powdered X-ray diffraction (XRD) techniques.

2. Materials and methods

Silibinin, chitosan and sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), trypsin–EDTA, antibiotic antimycotic solution, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Foetal bovine serum (FBS) was purchased from Gibco, USA. Cell culture 96 well plates and plastic wares were obtained from Techno plastic products (CH-8219 Trasadingen, Switzerland). All other chemicals were of analytical grade.

2.1. Preparation of silibinin loaded chitosan nanoparticles (SCN)

Chitosan nanoparticles were prepared by ionic gelation method with different ratios of chitosan and TPP (Table 1). Chitosan was dissolved in 1% acetic acid solution and pH was adjusted to pH 4.8. SLB was dissolved in acetone and was added dropwise to chitosan solution with continuous stirring and then sonicated for 2 min at 65% amplitude using probe sonicator (Vibra Sonics, Biotech-North America). TPP solution was added dropwise in above solution and further stirred at 500 rpm for 3 h. Nanoparticle suspension was centrifuged at 15,000 rpm for 30 min. Supernatant was removed and nanoparticles were washed twice with distilled water. Nanoparticles were lyophilized using trehalose (5%, w/v) as cryoprotectant using (Modulyod-230, Thermo electron freeze dryer, USA). The instrument was set at −50 °C with vacuum pressure less than 1 mbar. Blank chitosan nanoparticles (BCN) were prepared without SLB.

2.2. Physicochemical characterizations of nanoparticles

2.2.1. Particle size and zeta potential

Blank and SLB loaded nanoparticles were characterized for particle diameter, polydispersity index (PDI) and zeta potential by using Zetasizer, Nano-ZS (Malvern Instruments, UK). Before the analysis, nanoparticles dispersions were diluted with distilled water.

2.2.2. Transmission electron microscopy (TEM)

The surface morphology of SCN was examined by TEM analysis. Nanoparticles were dispersed in distilled water and a drop of the suspension was casted onto a copper grid. Extra solution was removed by filter paper, air-dried and directly observed under the transmission electron microscope (FEI Tecnai, G112, Philips, USA) at room temperature without staining.

2.2.3. Fourier transform infrared (FTIR) analysis

FTIR analysis was carried out for SLB, chitosan and SCN by using potassium bromide pellet method. Samples were mixed with potassium bromide, compressed into pellets and scanned for percent transmittance in the range of 4000–450 cm^{-1} using FTIR spectrophotometer (Perkin Elmer, Spectrum One, UK).

2.2.4. Differential scanning calorimetry (DSC)

Thermal behaviour of SLB, BCN, and SCN was performed by using DSC (EXSTAR DSC7020, Japan). The samples were heated in hermetically sealed aluminium pans at a scanning rate of 10 °C/min from 50 to 250 °C under inert nitrogen atmosphere. An empty aluminium pan was used as a reference.

2.2.5. Powder X-ray diffraction study (PXRD)

The X-ray diffraction patterns of SLB, BCN and SCN were obtained by using Siemens D-5000 X-ray diffractometer (Germany) with Cu Kα radiation generated at 45 kV and 20 mA. The diffraction patterns run over the range of 2θ from 2° to 60°, at steps of 0.013° with continuing time 13.6 s.

2.3. Silibinin entrapment efficiency

The entrapment efficiency (EE) was calculated by indirect method. SCN was centrifuged at 15,000 rpm for 30 min. Supernatant was collected and analyzed for silibinin content at 287 nm by using UV/VIS spectrophotometer (Perkin Elmer, Lambda 25, UK). The % EE was calculated as follows:

$$\%\ \text{EE} = \frac{\text{Amount of SLB added} - \text{Amount of SLB in supernatant}}{\text{Amount of SLB added}} \times 100$$

2.4. Dissolution study

Dissolution studies of pure SLB and SCN were carried out by using Dissolution test Station USP apparatus-2 (paddle type) (SR8 plus, Hanson Research, USA). The studies were carried out in two buffer mediums namely simulated gastric fluid (pH 1.2) and phosphate buffer (pH 6.8). A volume of 900 ml of buffer solution was placed in dissolution flasks and maintained at a constant temperature of 37 ± 0.5 °C. The apparatus was set at a speed of 100 rpm and 10 mg SLB or SCN (equivalent to 10 mg SLB) was added to individual dissolution flasks. An aliquot of 5 ml of sample was withdrawn at predetermined time intervals and equal volume of fresh buffer was added to maintain the sink conditions. Silibinin concentration in the samples was determined by UV/VIS spectrophotometer at 287 nm against the buffer solution.

2.5. Cell culture and preparation of test samples

Human prostate cancer cell line (DU-145) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were grown in DMEM medium supplemented with 10% FBS, 0.3% sodium bicarbonate, 10 ml/L antibiotic antimycotic solution (10,000 U/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B), 1 ml of 2 mM L-glutamine and 1 ml of 100 mM sodium pyruvate. The culture was maintained in CO_2 incubator at 37 °C with a 90% humidified atmosphere and 5% CO_2.

For the preparation of stock solutions of test compounds, pure SLB was dissolved in DMSO (1 mg/ml) whereas SCN was dispersed in sterile PBS. Various further dilutions were made with sterile PBS to get desired concentrations. All formulations were filtered with 0.22 μm sterile filter and were kept under UV radiation for 20 min before using.