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Preparation of collagen peptide functionalized chitosan nanoparticles by ionic gelation method: An effective carrier system for encapsulation and release of doxorubicin for cancer drug delivery

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ABSTRACT

In recent years, nanoparticles (NPs) based on biopolymers or peptides are gaining popularity for the encapsulation and release of drug molecules, especially for cancer therapy, due to their ability for targeted and controlled release. The use of collagen peptide (CP) for the preparation of chitosan (CN) NPs is especially interesting as it results in NPs that are stable under physiological conditions. In this work, mono-dispersed pH responsive CPCN NPs of about 100 nm were prepared via ionic gelation method by simple and mild co-precipitation of CN and CP. Investigation of NPs with Fourier transform infra-red (FTIR) spectroscopy and dynamic light scattering (DLS) measurements reveals that hydrogen bonding and electrostatic interactions are believed to be major driving forces for NP formation and drug encapsulation, respectively. Scanning electron microscopic (SEM) investigations show that hard and fine CPCN NPs transform to soft and bigger gel like particles as a function of collagen concentration. The unique "polymeric gel" structure of NPs showed high encapsulation efficiency towards doxorubicin hydrochloride (DOX) as well as pH controlled release. Anti-proliferative and cell viability analysis revealed that DOX loaded NPs showed excellent anti-proliferative characteristics against HeLa cells with favorable biocompatibility against normal cells. Such NPs have high potential for use as smart drug delivery carriers in advanced cancer therapy.

1. Introduction

Chitosan nanoparticles (CN NPs) are being used extensively for drug delivery applications because of its favorable properties such as biocompatibility, biodegradability and ability to bind with organic compounds, susceptibility to enzymatic hydrolysis, and intrinsic physiological activity [1–3]. The drug encapsulation and release characteristics of CN NPs are largely dependent on their size, surface potential and stability [3,4]. However, CN NPs suffer from poor solubility at a physiological pH of 7.4 due to the protonation of amino groups of CN, which causes presystemic metabolism of loaded drugs in intestinal and gastric fluids in the presence of proteolytic enzymes [3]. Another limitation of CN NPs arises from its tendency to absorb water and higher degree of swelling in aqueous environments, which leads to a burst release of loaded molecules. As a result, many forms of chemically modified (carboxylated, conjugated, thiolated, and acylated) CN derivatives have been prepared and used for drug delivery applications [5,6]. However, the chemical modification not only induces cytotoxicity issues, but also maintains CN molecules in precipitated and modified form; hence it might affect stimuli-responsiveness of the resulting NPs as well as encapsulation and release behavior of NPs. To circumvent these limitations, biomolecules like caseino-phosphopeptide can be used for CN NP synthesis [7]. CN NPs functionalized with peptides have shown improved properties such as high stability, low degree of swelling, high bioavailability, sustained release of loaded molecules and low cytotoxicity [7,8]. For instance, encapsulation of (−)-epigallocatechin-3-gallate (EGCG) in food grade nanoparticles of chitosan caseinophosphopeptide improved nanochemoprevenion of EGCG when it was administered orally [7].

Even though different methods are reported for the fabrication of CN NPs [9–12], the ionic gelation method is widely used for the fabrication of CN NPs due to the following reasons: a) use of water based solutions; b) high stability of synthesized particles; c) mild reaction conditions without organic solvents and d) simple and cost effective process. The high stability of CN NPs in the ionic gelation method can be partially attributed to the ionic cross-linking of positively charged CN molecules with negatively charged polyanions [13]. The most commonly used polyanion for the ionic cross-linking of CN is tripolyphosphate (TPP), which is nontoxic [14,15]. The parameters such as molar ratio of TPP and CN, their interaction mechanisms and process conditions (e.g. temperature and pH) play a key role in the control of size and morphology of the NPs [16–18]. Recently, it is reported that CN films incorporated

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with collagen (CL), a natural biomolecule, showed improved morphological stability at physiological conditions [19,20]. The stability is increased as a function of CL concentration up to certain extent, and then starts to decrease as excess amine groups available at higher concentration lead to morphological instability by inducing electrostatic repulsion with neighboring amine groups [20–22]. The additional benefit of using CL in CN films is that it can preserve the native properties of CN when compared to chemical methods that alter bulk properties of CN. Though CLCN film formation and its use in drug delivery were reported earlier, preparation of collagen peptide–chitosan (CPCN) NPs and investigation of underlying interaction mechanisms on NP size and morphology are not yet explored. Further, the driving forces (e.g. hydrogen bonding or electrostatic interactions) that are responsible for particle formation as well as drug encapsulation need to be investigated in detail since these forces can significantly affect the morphology, stability and release behavior of NPs.

In this study, we have prepared mono-dispersed pH responsive CPCN NPs via ionic gelation method by simple and mild co-precipitation of CN and CP. The potential of synthesized NPs for encapsulation and release of DOX for cancer drug delivery was also investigated. We also intend to provide the information about polymer–polymer and DOX–NP interactions to investigate the mechanisms behind the particle formation as well as drug encapsulation and release process. Hence, the proposed study not only extends our knowledge on CPCN NP formation, but also provides an effective drug delivery system for encapsulation and release of water soluble drugs.

2. Materials and methods

2.1. Materials

All reagents and chemicals used were of analytical grade. Chitosan (CN) (M_W = 190–310 kDa; viscosity = 200–800 cP and degree of deacetylation = 75–85%), tripolyphosphate (TPP) and doxorubicin hydrochloride (DOX) were purchased from Sigma-Aldrich, India. Collagen peptide (CP) was purchased from HiMedia Laboratories, Mumbai, India. All other reagents and chemicals used for the study were bought from SRL Ltd., India. Milli-Q water with resistivity >18 MΩ cm was used for all the experiments.

2.2. Preparation of CN and CPCN NPs

CN NPs were prepared by ionic cross-linking of CN solution with TPP as a polyanion [13]. Briefly, CN and TPP were dissolved in 1% acetic acid and water to obtain stock solutions of 5 mg/mL and 8.4 mg/mL, respectively. CN NPs were prepared by adding 5 mL of TPP solution (0.84 mg/mL) into 10 mL of CN solution (2 mg/mL) under ultrasonication at room temperature. Hence, the final concentrations of CN and TPP were 1.33 mg/mL and 0.28 mg/mL, respectively. The concentrations of CN and TPP were chosen based on the ranges reported to produce opalescent suspensions of very small NPs [23]. Volumetric composition and other details are given in the Supplementary data (Tables 1–2). For the preparation of CPCN NPs, CP stock solution of 5% (*w*/*v*) was prepared in water and added along with TPP into CN solutions under ultrasonication at room temperature. It is noteworthy that solutions (TPP without or with CP) were added in a drop wise manner into the chitosan solution to synthesize CN or CPCN NPs. To investigate the influence of CP concentration on NP size and morphology, the final concentration of CP was varied from 0 to 2%. After mixing, the pH of the suspension was adjusted to 7.0 by adding 0.1 M NaOH and the stirring was continued for another 30 min. Without pH adjustment, synthesis at pH 3.5 (pH of reaction mixture) results in NPs with protonated amino groups [23,24], hence, it might affect the encapsulation of cationic drug molecules like DOX. The formation of a turbid solution confirms the formation of CPCN NPs. The formed particles were separated by centrifugation at 12,000 rpm and washed three times with water. After preparation, NPs were freeze dried and stored in a dark atmosphere at 4 °C for characterization.

Adding a DOX solution of 1 mg/mL in a drop wise manner during the preparation of CPCN NPs (after changing the pH to 7) resulted in DOX loaded CPCN NPs. The particles were then washed, freeze dried and stored in a dark atmosphere at 4 °C for characterization and release experiments.

2.3. Characterization

The average particle size and zeta potential (ZP) of CPCN NPs were determined by a dynamic light scattering (DLS) technique using a Zeta sizerNano ZS (Malvern Instruments, U.K.), at room temperature. The measurements were performed at pH 7, at which the influence of pH on NP size is minimum. Hence, it can be compared with the size of DOX loaded CPCN NPs. Before measurements, the NP suspension was diluted with water (1 part is added with 4 parts of water) and subjected to ultrasonication for 2 min to avoid aggregation of NPs.

The crystalline states of CPCN NPs were evaluated by X-ray powder diffraction (XRD) analysis (Philips X'Pert X-ray Diffractometer, Philips Innovation Services, Netherlands). The X-ray generator was operated at 40 kV tube voltage and 40 mA of tube current, using Kα lines of copper as the radiation source. The scanning angle was changed from 1 to 60° for 1 h in a step scan mode (step width of 1°/min).

For FTIR analysis, 5 mg of dried NPs was mixed with KBr powder and made into pellets. Spectra were acquired in transmission mode on a Nicolet 5700 FTIR spectrometer (Thermo Electron Corporation, USA).

For SEM analysis, a drop of the NP suspension was placed on a silicon wafer and air-dried overnight to remove the moisture completely. After sputtering a thin gold layer, NPs were analyzed using a field emission SEM (FEI-SIRION, Eindhoven, Netherlands).

2.4. Determination of encapsulation efficiency (EE)

The amount of DOX loaded in NPs was estimated by subtracting the free DOX present in the supernatant from the original amount used for synthesis. To calculate unloaded (free) DOX, DOX loaded CPCN NPs were washed thrice with water and the amount of DOX present in the wash water was estimated by UV–visible spectroscopy (Nanodrop 2000c Spectrophotometer, Nanodrop Technologies, USA). The EE was calculated by the following equation:

$$\text{EE}\ (\%) = \frac{\text{Amount of drug loaded in CPCN NPs}}{\text{Initial amount of drug}} \times 100$$

2.5. In-vitro drug release experiments

The release experiments were performed in a dark room at 37 °C using phosphate buffered saline (PBS: pH 7.4, 0.14 M NaCl), acetate buffered saline (pH 5.5, 0.14 M NaCl) and HCl buffer (pH 1.5, 0.2 M KCl) as release medium by dialysis method. 50 mg of drug loaded NPs was loaded in a dialysis membrane tubing (molecular weight cut-off of 8000 Da) containing 3 mL of the appropriate buffer (donor solution) and dialyzed against 50 mL of the same buffer (receiver solution) in a standard flask kept in an incubator shaker at 37 °C. The flask was kept sealed from the ambient atmosphere to minimize the evaporative loss. At predetermined time points, 3 mL of the sample was extracted from the flask and replaced with 3 mL of pre-warmed buffer in a manner so as not to disturb the mechanical equilibrium. After 3 days, the receiver solution was completely removed from the flask and replaced with a fresh pre-warmed solution to avoid any precipitation of salts present in buffers. The amount of DOX released was estimated by measuring the increase in absorbance values at 480 nm using UV–visible spectroscopy.