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Original article

Effect of cryoprotection on particle size stability and preservation of chitosan nanoparticles with and without hyaluronate or alginate coating

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ABSTRACT

The aim of the present study was to determine the effect of different cryoprotectants and their concentration on the physicochemical characteristics of chitosan nanoparticles (CS-NPs). The effect of coating of CS-NPs with hyaluronic acid (HA) and alginic acid (ALG) before and after lyophilization was also evaluated. The ionic gelation method was used for the preparation of NPs and six different types of cryoprotectants (sucrose, glucose, trehalose, mannitol, polyethylene glycol-2000, and polyethylene glycol-10,000) were investigated at 5%, 10%, 20%, and 50% concentration levels. Coating of CS-NPs with HA and their protection with high amount of cryoprotectants indicated better particle size stability. Samples that were lyophilized without cryoprotectants resulted in an increase in average size due to high agglomeration. All cryoprotectants with varying amount provided some sort of size stability for the NPs except for the PEG-10,000 which had no protective effect at higher concentrations. Sucrose and trehalose sugars were found to have the highest protective effect with HA coated and uncoated CS-NPs. In conclusion, using cryoprotectants along with surface coating, the CS-NPs could achieve the desired physicochemical characteristics for a prolonged duration.

1. Introduction

Due to exceptional unique physical and biological characteristics, the polysaccharides have got prime attention as a carrier in drug delivery systems (Liu et al., 2008). Chitosan (CS) is a cationic polysaccharide derived from chitin which is a biopolymer, isolated from the exoskeleton of crustaceans, and some fungi (Sagheer et al., 2009). CS has drawn great attention in industrial areas such as paper technology, food, water filtration, agriculture, pharmaceuticals and biomedical industries (Jain et al., 2014). Use of CS for NPs preparation has been broadly employed for delivery of different kinds of payloads including drugs, proteins, peptides, genes, DNA, because of its numerous attractive features such as less immunogenic and relatively low toxic, excellent biocompatibility and biodegradability (Almalik et al., 2013a). The CS-NPs have found to have larger activated surface area than other physical forms of materials which support drug loading capacity, long shelf life, good permeability to epithelia and convenience of transporting into the body owing to its unique structure (Gokce et al., 2014). CS-NPs could be synthesizing by different methods such as ionic gelation, synthesizing with carboxymethyl cellulose using glutaraldehyde as cross-linker, with alginic acid or hyaluronic acid, coacervation, reverse micellar and polymerization with poly (hydroxyethyl methacrylate) techniques (Fabregas et al., 2013; Jain et al., 2014). Among these methods ionic gelation technique is widely preferred for the preparation of CS-NPs (de Pinho Neves et al., 2014). The formation of particles using ionic gelation process is somewhat mild and avoids demanding organic solvents and high temperatures allowing effective encompassing of delicate molecules without any damage and loss (Fan et al., 2012). This method principally depends up on ionic interactions between cationic chains of CS and anionic charged polyanions e.g. tripolyphosphate (TPP) as cross linker (Deng et al., 2014).

Besides encapsulation and drug loading CS-NP's could also be coated with certain materials to enhance their efficacy, better

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targeting and to make them more specific as a carrier (Almalik et al., 2013a). Polyionic nano-complexes (PICMs) composed of polycations and opposite polyanions, have been found to have a great potential in biomedical and nano-biotechnological applications including controlled drug release and gene transfection (Deng et al., 2014). Among numerous polyionic nano-complexes, hyaluronic acid based chitosan nanoparticles (HA-CS-NPs) and alginic acid based chitosan nanoparticles (ALG-CS-NPs) have been investigated fundamentally in recent years (Azevedo et al., 2014; Gokce et al., 2014). The coating of CS-NPs causes the conversion of surface charge of the NPs. HA and sodium-ALG have been found to provide negative charges on the surface of NPs as HA and ALG molecules are mainly present in the outer shell of the NPs (Borges et al., 2006). However positively charged nanocarriers enhance membrane association, internalization and endosomal escape, while those bearing a negative zeta potential has shown more specific and efficient uptake, especially when coated with targeting ligands (Almalik et al., 2013b).

The ionic gelation method causes the aggregation and fusion of NPs after synthesis when stored for long time which might be due to the restricted physicochemical stability of NP-suspensions (Gokce et al., 2014). Physical instability frequently occurs when NPs were stored for long term in aqueous medium which restricted their use (Rampino et al., 2013). Freeze drying of NP-suspension prevents the aggregation and fusion of particles also maintains the size-stability during long-term storage of NPs. However, if the NPs are lyophilized without any cryoprotectant the formation of aggregates can greatly hinder the re-dispersion of NPs, and thus affect their sizes (Fabregas et al., 2013). Similarly, CS-NPs form aggregation after freeze-drying due to inter-and intramolecular hydrogen bonding unless the cryoprotectant is used (Abdelwahed et al., 2006). Hence, cryoprotectants must be used during the freeze-drying of CS-NPs to remove excess moisture and increase long-term storage and to protect the CS-NPs size stability. The use of trehalose, mannitol, sorbitol or glycerol as cryoprotectant is an efficient way during freeze-drying of NPs to maintain their physical properties (Gokce et al., 2014). The purpose of this study was first to develop coated and uncoated CS-NPs by ionic gelation method. The desired sized coated CS-NPs were developed with the ideal formulation conditions. After their synthesis, the CS-NPs were mixed with different cryoprotectants at different ratios and lyophilized to get the freeze-dried product of CS-NPs. The CS-NPs were further subjected to physicochemical characterization by using zeta-sizer to measure particle size, their population, and size distribution before and after lyophilization to determine the best suitable cryoprotectants and their optimum combination or mixture to get the best sized and stable CS-NPs.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree over 60% mol, from white mushrooms; Sigma, UK), 1 M hydrochloric acid (HCl), 1 M sodium hydroxide (NaOH) and sodium triphosphate pentabasic (TPP) were obtained from Sigma–Aldrich (Gillingham, UK); hyaluronic acid (HA) 200 kDa was obtained from Medipol SA (Lausanne, Switzerland) and Sodium Alginate (400 kDa) was obtained purchased from Sigma, UK. Glacial acetic acid and sodium acetate were purchased from VWR, BDH Chemicals (Poole, UK). Trehalose dihydrate was obtained from Merck (Darmstadt, Germany). Sucrose, glucose, mannitol, polyethylene glycols as PEG 2,000 and PEG 10,000 were obtained from Sigma-Aldrich Co. (St. Louis, Missouri). The RC-dialysis membrane of MWCO 10 kDa was obtained from (Spectra Por, Spectrum Laboratories Inc., Rancho Dominguez CA, USA). The other chemicals and reagents were of AR grade and were used as received.

2.2. Formulation of uncoated CS-NPs

The CS-NPs were prepared by ionic gelation method using tripolyphosphate (TPP) as crosslinking agent. Accurately 0.069%w CS solution was prepared by dissolving purified CS in 4.6 mM HCl and the pH of this solution was adjusted to 5 by the addition of appropriate volumes of 0.1 M NaOH. The obtained solution was kept on magnetic stirring overnight prior to use. A 0.1%w tripolyphosphate (TPP) was dissolved in deionized water and the pH of the solution was maintained to 5 with 0.1 M HCl. Both the solutions were filtered through a 0.22 μm size Millipore® filter. Exactly 215 μL of the TPP solution was added to the CS-solution to get a final volume of 3 mL leading to 9:1 CS: TPP mass ratio and the actual concentrations of CS and TPP in terms of percentage weight (%w), reached to 0.064 and 0.0071 respectively (Almalik et al., 2013a).

2.3. Development of HA and ALG-coated NPs

The prepared CS-NPs were subjected for coating with HA and ALG. Briefly, chitosan-TPP NPs were dispersed at a concentration of 0.025 weight percent (wt%) in 0.1 M acetic acid/acetate buffer at pH 5 and mixed by using magnetic stirring (500 rpm for 15 min). The dispersions were then slowly added under the continuous and vigorous stirring at 1200 rpm for 30 min to an equal amount and an equal strength of acetate buffer, containing HA (200 KDa) and ALG separately at a concentration of 1.5 mg mL^{-1} (Almalik et al., 2013b). The obtained dispersions were then dialyzed against deionized water by using dialysis membrane (MWCO = 10 KDa). Then, equal volumes of HA-coated and uncoated CS-NPs in deionized water were lyophilized and reweighted, and the actual dry weight for HA-coated CS-NPs was roughly double than that of the uncoated CS-NPs, suggesting a 0.5 HA weight fraction (Almalik et al., 2013a).

2.4. Particle size, polydispersity and zeta potential measurement

Hydrodynamic diameter (*Z*-average), polydispersity (PDI) and zeta potential measurements were always measured on three independent samples of the CS-NPs at 25 °C temperature using a Malvern Zetasizer Nano-ZS (ZEN3600, Malvern Instruments Ltd, UK) equipped with a solid state HeNe laser (633 nm λ_{max}) at 173 degree scattering angle. The hydrodynamic diameter and particle size distribution were determined by Dynamic Light Scattering (DLS) also known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS) which is the most popular light scattering technique as it permits particle sizing even down to a diameter of 1 nm. Dynamic light scattering technique determines the diffusivity of the particles in the suspension based on the time-dependent fluctuations in the intensity of scattered light resulting from the Brownian motion. Size of the particles is therefore calculated from the Stokes-Einstein equation.

2.5. Lyophilization of CS-NPs and their recovery

The freshly prepared CS-NPs were filtered through 0.22 μm hydrophilic Millipore® syringe filters (to remove the lumps formed during ionic gelation of CS and TPP) and subjected to the process of lyophilization by using FreeZone Triad Cascade Benchtop Freeze Dry System (Labconco, Missouri, USA). About 1 mL of each ALG and HA coated CS-NPs suspensions, was freeze-dried with and without using cryoprotectants (glucose, sucrose, trehalose, mannitol, PEG 2000, or PEG 10,000) at 5%, 10%, 20%, and 50%,