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Nanoparticles as a novel delivery system for vitamin C administration in aquaculture

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ABSTRACT

The potential of chitosan-based nanoencapsulation as a tool for delivering ascorbic acid (AA) to marine and freshwater organisms was investigated. Polymeric non-loaded and loaded vitamin C nanoparticles (NPs) were made by ionic gelation and the particles were characterized. In vitro performance of nanoparticles was evaluated in a zebrafish liver cell-line (ZFL) and in vivo studies were carried out in fish (post-metamorphic larvae of *Solea senegalensis*) and rotifers (*Brachionus plicatilis*) to assess the potential use of these NPs to be used as a tool in nutritional aquaculture studies. The results showed that NPs are suitable to trap hydrosoluble compounds such as AA by forming positively charged complexes (30–35 mV), in a nanosize range (<300 nm), with encapsulation efficiency (EE) higher than 15% and high stability (>90% of loaded AA remained within nanoparticles after 2 h in seawater). The potential cytotoxicity of the NPs was evaluated in ZFL cells and no decrease in cell viability was noted up to 2.5 mg/ml of nanoparticle concentration. The NP uptake was analyzed in ZFL cells by FACS cytometry and confocal laser scanning microscopy (CLSM). Time course and dose–response experiments were performed using fluorescein isothiocyanate labeled NPs (FITC-NPs). The in vitro endocytosis assays with ZFL cells showed a maximum uptake after 6 h of incubation and a dose-dependent increase of fluorescence intensity directly proportional to the FITC-NP concentration. The antioxidant properties of vitamin C nanoparticles (AA-NPs) were also analyzed in ZFL cell extracts. Lipopolysaccharide (LPS) was added to ZFL cells to induce oxidative stress. The total antioxidant capacity of the AA-NP-treated cells showed a statistically significant increase with respect to the control with non-loaded nanoparticles (71.00 ± 9.6 and 25.36 ± 3.96 μM Trolox equivalent; $p < 0.05$ respectively). The NPs' ability to penetrate fish intestinal epithelium was also evaluated. After 2 h, NPs were able to penetrate through intestinal epithelium in post-metamorphic larvae of *S. senegalensis* as detected by CLSM. The potential use of NPs as additive to rotifers was analyzed using AA-loaded or non-loaded NPs in rotifer enrichment for 2 h. Rotifers fed with AA-NPs increased up to 2-fold of their ascorbic acid levels in comparison to control groups. As a whole, results show that these polymeric NPs might represent an interesting vehicle for oral administration of AA and other active compounds in aquaculture.

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1. Introduction

The field of nanotechnology has experienced a significant increase over the last years, but few studies have focused on nanotechnology applications in the aquaculture area. Thus, although the application of the nanotechnology to aquaculture is still at a very early stage, it may have the potential to solve many puzzles related to animal health, production, reproduction, as well as prevention and treatment of diseases.

One particular advantage of nanoencapsulation is to provide a more effective method to supply unstable and/or hydrosoluble micronutrients and therefore be used as a tool for studies of nutritional requirements in aquaculture. One of these soluble and labile compounds is the vitamin C (ascorbic acid, AA), which is an essential micronutrient for marine fish and plays a key role in a wide range of physiological processes, including growth, reproduction, response to stressors and immune response (Carr and Frei, 1999; Corti et al., 2010). However, some mammals including human, and also all teleost fishes cannot synthesize AA due to the lack of L-gulonolactone oxidase (EC 1.1.3.8), which is the key enzyme for the de novo synthesis of AA (Moreau and Dabrowski, 2001). Ascorbic acid is

Abbreviations: NPs, nanoparticles; CS, chitosan; CDs, cyclodextrins; AA, ascorbic acid; DD, deacetylation degree; CM, carboxymethyl; TPP, sodium tripolyphosphate; LMW, low molecular weight; HPLC, high performance liquid chromatography; EE, encapsulation efficiency; LC, loading capacity; Y, nanoparticle yield; MPA, metaphosphoric acid; EDTA, ethylenediaminetetraacetic acid; Z, zeta potential; FITC, fluorescein isothiocyanate labeled; ZFL, zebrafish liver cell line; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); LDH, lactate dehydrogenase activity; FACS, fluorescence-activated cell sorting; CLSM, confocal laser scanning microscopy; SEM, scanning electron microscopy; MFI, geometric mean fluorescence intensity.

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highly labile and most of its functionality is lost during processing and storage of food and feeds because of the exposure to high temperature, oxygen and light (Soliman et al., 1987). Shiau and Hsu (1994) found that approximately 75% of the initial amount of supplemented vitamin C in shrimp feeds was lost during its processing at room temperature.

Studies on specific vitamin C requirements are scarce in marine fish larvae due to its poor retention in inert foods and the lost of its functionality in water. It is important to develop a dietary particle that can incorporate and deliver water-soluble nutrients to fish larvae meanwhile there are severe problems with this kind of nutrients due to leaching (Kvåle et al., 2006; Lopez-Alvarado et al., 1994). Several researchers have investigated the use of different methodologies to make beads to deliver water-soluble substances to marine suspension feeders (Buchal and Langdon, 1998; Nordgreen et al., 2008; Önal and Langdon, 2004) and although some improvements have been achieved (Önal and Langdon, 2004; Yúfera et al., 2002), substantial losses of these water-soluble substances, such as vitamin C, occur during food preparation (Langdon et al., 2008; Nordgreen et al., 2008). The use of more stable forms of vitamin C is also a crucial requirement in this field. Nanoencapsulation appears as a suitable approach to enhance vitamin C stability and it is presumed to be an efficient method that allows homogeneous distribution within the organism while maintaining all its chemical and nutritional properties (Alishahi et al., 2011).

Recently, there has been growing interest in delivering systems prepared by ionic gelation because of its very simple and mild preparation conditions, homogeneous particle sizes and bioadhesive properties. Furthermore, a new generation of hybrid polysaccharide nanocarriers composed of chitosan and anionic cyclodextrin is proposed to combine the promising behavior of chitosan nanoparticles (NPs) with the excellent biopharmaceutical properties of cyclodextrin (Teijeiro-Osorio et al., 2009).

In order to nanoencapsulate water-soluble compounds, one of the possible strategies is to use chitosan–cyclodextrin NPs synthesized by ionic gelation. Chitosan, is a natural biopolymer that has been widely used as an oral delivery system for many bioactive agents due to its nontoxic, biocompatible, and biodegradable characteristics (Agnihotri et al., 2004). Chitosan has also good bioadhesive properties, therefore a chitosan delivery system would provide bioactive agents with much longer residence time in the gastrointestinal track, thereby resulting in improved bioavailability (Bernkop-Schnürcha et al., 2004; Sinha et al., 2004). On the other hand, cyclodextrin is a compound that has been extensively used due to its special ability to complex with a variety of molecules and to enable their solubility, stability, bioavailability and protection against oxidation (Challa et al., 2005; Cryan et al., 2004).

The overall goal of this work was to assess the potential of NPs based on biodegradable and non-toxic materials as a vehicle for the delivery of vitamin C to aquatic organisms. To reach this goal different aspects need to be taken into consideration: i) the materials used to make the NPs must be safe and the particles have to be nanosized, with an appropriate charge in order to avoid aggregation; ii) the final application of these NPs is to be used in aquaculture, therefore it is important to determine its stability under real conditions of pH (7.5–8), temperature (20 ºC) and salinity; and iii) the cell uptake capacity and in vivo target tissues and uptake.

To date, little information is available about the bioavailability, uptake and distribution of polymeric nanoparticles in aquatic organisms and their effects. In this context, the aim of this study was to elaborate chitosan–cyclodextrin NPs loaded with vitamin C in order to determine their properties, cytotoxicity and uptake in cells and tissues of different aquatic organisms. Several key features of these NPs including size, zeta potential, entrapment efficiency and seawater stability were studied. In addition, the in vitro and in vivo performances of these NPs were assessed in a zebrafish liver cell line (ZFL) and in post-metamorphic sole larvae and rotifers, respectively. This work contributes to assess the use of NPs in aquaculture and may help in understanding the nutritional requirements of marine and freshwater suspension-feeder, including larvae of many fish species.

2. Materials and methods

2.1. Materials

Chitosan (CS) with a 75% deacetylation degree (DD) and low molecular weight (LMW); cyclodextrin (CD) anionic derivative, carboxymethyl–β-CD (CM–β-CD), sodium tripolyphosphate (TPP) technical grade 85%, sodium L-ascorbate (AA), mouse EGF, DMSO cell culture tested, Antioxidant Assay Kit, PBS10× sterile and endotoxin free water were all purchased from Sigma-Aldrich (Spain). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA laboratories. Antibiotic/antimycotic and TrypEL Express were purchased from Gibco. Trout serum was obtained as described in Ruyra et al. (2013). All plasticware was purchased from Beckton–Dickinson and the Cytotoxicity LDH assay Kit was from Roche. Ultrapure water, and other solvent and chemicals were of the highest grade commercially available.

2.2. Preparation of NPs

Chitosan–CD–TPP NPs were prepared by ionotropic gelation as described by Teijeiro-Osorio et al. (2009) and Jang and Lee (2008) with some modifications. Briefly, chitosan was dissolved at a concentration of 2.4 mg/ml in acetic acid solution (0.4% v/v) and filtered through 0.45 μm. AA was added (0.36 mg/ml) to the CD–TPP solution under magnetic stirring at room temperature. AA–CD–TPP solution was then added to the CS solution using a peristaltic pump at a flow rate of 1 ml/min in order to obtain a 4/3/0.6 ratio. Magnetic stirring was maintained for 15 min to allow the complete formation of the system. Particles were isolated by centrifugation at 4 ºC for 60 min at 40,000 ×*g*. The supernatant was collected for determination of non-encapsulated AA. Finally, NPs were freeze-dried and stored at 4 ºC until use.

2.3. Determination of vitamin C by HPLC

After NP preparation, the supernatant was removed and the vitamin C extracted with 6% metaphosphoric acid (MPA) containing 2 mM EDTA. 1 ml of extraction buffer was added to 1 ml of supernatant and the samples were then homogenized for 30 s with a vortex, keeping them on ice and protected from light. Then, the samples were passed through a nylon filter (0.45 μm) and the AA content was analyzed on a Finnigan Surveyor Plus HPLC system (Thermo Scientific), equipped with an autosampler and a photo diode array detector. Compounds were separated on a Hipersil Gold C18 (5 μm) column. The temperature of the autosampler was 4 °C and the column temperature was 30 °C. The elution was performed isocratically with 1% NaH_2PO_4 at pH 2.7 with a flow rate of 0.8 ml/min.

2.4. Physico-chemical characterization of the NPs

Particle size and zeta potential measurements (ζ′) were determined by dynamic light scattering and by laser Doppler electrophoresis respectively, using a Zetasizer Nano ZS (Malvern Instruments, UK). Particle size measurements were carried out in a standard folded cell (Polystyrene 10 × 10 × 50, Sarstedt, Malvern Instruments) at 20 ºC using PBS at pH 7.4 as a dilution buffer (1/10). Zeta potential measurements were performed in a folded capillary electrophoresis cell (Malvern Instruments) at 20 ºC using a 0.1 M KCl solution as dilution buffer. Data analysis of NP size and zeta potential is presented as mean and standard deviation of triplicate runs. The morphology of NPs was evaluated by a transmission electron microscopy (TEM) using a PHILIPS CM-200 and by a scanning electron microscopy (SEM) using a FEI Quanta 200.

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