P-079 **A comparative study of two nanoencapsulation techniques to trap vitamin C to aquaculture application.**

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INTRODUCTION AND OBJECTIVES

Despite several decades of research, there is still a lack of understanding on the nutritional requirements of marine fish larvae. One reason is related with technical difficulties in delivering water-soluble micronutrients. According to Holt et al, (2011), it is necessary apply new technologies to diet development to solve deficiencies in larval nutrition and to advance in weaning. The development and application of nanoencapsulation techniques offer the opportunity to obtain tiny particles that are capable of carrying water-soluble ingredients of diet such as ascorbic acid (AA). Different preparation methods may permit special characteristics suitable for certain class of compound and associated delivering conditions. Ascorbic acid (AA) is a water-soluble vitamin essential to marine fish. This vitamin is present in a variety of biological functions such as promoting collagen biosynthesis, scavenging of free radicals and immunity enhancement.

The aim of this work was to assess the potential of two different methods based on biodegradable and non-toxic polysaccharides as a tool to provide vitamin C to marine organism in aquaculture conditions. For this purpose, we prepared Chitosan Nanoparticles (CS) and AA-loaded CS nanoparticles (CS-AA) by the ionotropic gelification of CS with tripolyphosphates and cyclodextrins anions (CD-TPP). On the other hand, chitosan-alginate nanoparticles were prepared without (CS-ALG) or with ascorbic acid (CS-ALG-AA) by precipitation/coacervation methodology. We evaluated suitability of each method to be used in aquaculture by particle size, zeta potential, encapsulation efficiency (EE) and yield measurements. Also, nanoparticles stability was evaluated during 2h in seawater solutions.

MATERIALS AND METHODS

Chitosan-Alginate (CS-ALG) particles were prepared as described by Borges et al, (2005) by precipitation/coacervation method, with some modifications. Thus, Low MW chitosan (0.25% (w/v) was dissolved in aqueous acetic acid solution (1% v/v) and filtered through a 0,45mm membrane. After complete dissolution, Tween® 80 (1% w/v) was added. The formation of nanoparticles was achieved after the addition of 3.5 ml of sodium sulfate solution (10%)(w/v) to 200 ml of chitosan solution. Particles were isolated using centrifugation and two Milli-Q water washing. Then, the supernatant was discarded and the precipitate was freeze-dried. CS-ALG-AA particles were obtained by incubation of CS-ALG (dry powder) with a CS/AA ratio (100/0; 75/25; 50/50; 25/75) in phosphate buffer pH 7,4. After that, an alginate $CaCl_2$ coated (chitosan-Alginate ratio: 1/4) was performed. The supernatant was analyzed by HPLC and the precipitate was freeze-dried. Chitosan-TPP (CS-TPP) nanoparticles were prepared by ionic gelification as described Teijeiro-Osorio et al, (2009) and Jang et al, (2008) with some modifications. Briefly, chitosan (Medium MW) at 2,4mg/mL was dissolved in acetic acid solution (0,4%v/v) and filtered through 0,45µm. In 25mL of this solution, AA was dissolved at different CS/AA ratios (100/0; 85/15; 25/75; 50/50) followed by the addition of 15 mL of CD/TPP at flow of 1mL/min. The final ratio CS/CD/TPP used was 4/3/1,6. Particle size measurements were performed by dynamic light scattering and Zeta potential measurement by laser doppler electrophoresis using for both cases a Zetasizer Nano ZS (Malvern Instrument, UK). For determination of AA encapsulation efficiency, nanoparticles were isolated by centrifugation and the supernatant was analyzed by HPLC as described previously by Alishahi et al (2011) and Davey, et al (2003). The AA released from CS-ALG-AA and CS-TPP-AA nanoparticles was performed in seawater conditions (SW) salinity of 30ppt, 20ºC for 2 hours).

RESULTS AND DISCUSSION

Similar AA encapsulation efficiency (EE), around 20%, for nanoparticles prepared by the CS-TPP was found. When CS-ALG method was used the highest EE load AA (17,73%±3,38) was obtained at 25/75 (CS/AA). Table 1 shows size, the polidispersion index and Z-potential of different nanoparticles as the methodology used in the present work. Higher particle size (around 600nm) for CS-ALG nanoparticles was obtained. An adequate polidispersion index with a narrow distribution (around 0,45) was found for both particle types. Similar particle size (around 300nm) and different load of AA were found for CS-TPP nanoparticles. Nanoparticles positive or negatively charged were obtained using CS-TPP or CS-ALG methodology, respectively. Zeta (Z) potential of CS-TPP nanoparticles was above +20mV corresponding with that found by other authors (Krauland and Alonso 2007). In CS-ALG nanoparticles, Z potential had a negative value lower than -30 mV, thus these nanoparticles have higher stability in suspension than CS-TPP nanoparticles. Nanoparticle uptake in mucosal and epithelial tissue, and intracellular trafficking is controlled by their size and surface charge (Ravi Kumar

2004; Khatri, Goyal et al. 2008; Keum-Il Jang, 2008). Small mean particle size, positive charge and uniform particle distribution observed in CS-TPP-AA could be associated with uptake in larvae intestinal cells through mucosal barriers with higher mucoadhesivity.

Table1:Particle size;Polidispersion index(PI);Zpotential[mean±sd]

CS/AA	Particle Size (nm)	PI	Zpotential (mV)
CS-TPP			
100/0	335,6±17,7	0,45±0,02	18,5±0,28
85/15	310±19,6	0,41±0,00	20,55±0,21
75/25	282,75±9,8	0,48±0,06	20,95±0,50
50/50	331,45±9,4	0,56±0,04	19,25±0,21
CS-ALG			
100/0	568,6±32,1	0,52±0,00	-55,55±1,63
75/25	716,5±63,6	0,50±0,08	-42,85±1,48
50/50	690±305	0,39±0,12	-53,2±0,99
25/75	599,6±64,2	0,47±0,12	-55,7±0,85

Release studies Both methodologies used in present study resulted in stable nanoparticles remaining more than 88% of vitamin C loaded after two hours in seawater conditions. CS-TPP-AA nanoparticles prepared with a CS/AA ratio of 85/15 showed higher stability (Figure 1). More stable CS-ALG nanoparticles retain more AA, above 90%. Initial AA release might be adsorbed at the surface of nanoparticles that immediately dissolves in contact with seawater. For CS-TPP particles, the lowest initial release was found for the batch prepared with the highest CS/AA ratio. On the contrary, the same batch in CS-ALG particles achieved the highest initial value of release. No significant differences were found for nanoparticles regarding the method and other CS/AA ratios.

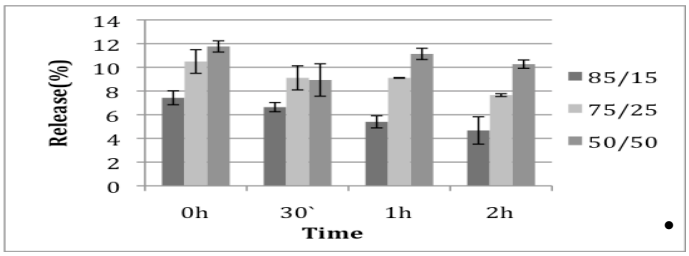

Figure 1: AA Release from CS-TPP particle in SW. NPs were loaded with differents CS/AA ratios

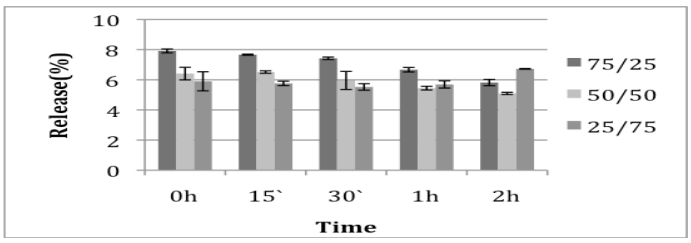

Figure 2: AA Release from CS-ALG **particle in SW. NPs were loaded with different CS/AA ratios**

CONCLUSIONS

Chitosan is a biodegradable material used in nanoparticles elaboration with adequate properties to be used in aquaculture. Both methodologies, precipitation/coacervation and ionic gelification, evaluated in the present study are suitable to trap ascorbic acid and showed to have potential to be used as useful tool in aquaculture nutrition studies. Ascorbic acid-loaded nanoparticles showed a good stability in seawater conditions. Positive charge, low size nanoparticles, together with stability property in seawater are suitable characteristic that suggest the usefulness of CS-TPP method for aquaculture nutrition studies. After two hours in seawater conditions at 20ºC, at least 85% of vitamin C remains within nanoparticles. It is time enough to be available for rotifers or larvae in a standard enrichment. We are currently studying the response of rotifers procedure as larval prey, to these nanoparticles. Nanoencapsulation is a novel technology with many promising applications emerging for the aquaculture area and more research in this way is necessary.

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