Food Chemistry 126 (2011) 935–940

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Shelf life and delivery enhancement of vitamin C using chitosan nanoparticles

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ARTICLE INFO

Article history:
Received 23 June 2010
Received in revised form 28 October 2010
Accepted 12 November 2010
Available online 20 November 2010

Keywords:
Chitosan nanoparticles
Vitamin C encapsulation
Shelf life
Controlled release

ABSTRACT

Chitosan with different molecular masses was reacted with sodium tripolyphosphate (STPP) to prepare different size nanoparticles, in which vitamin C was encapsulated. The effect of molecular weight (Mw) on nanoparticles efficiency, nanoparticles yield, size, and zeta potential was investigated in detail. Low Mw chitosan generated nanoparticles with better size, morphology, and delivery rate. In addition, the shelf life of encapsulated vitamin C increased as compared with its non-encapsulated counterpart. The release of vitamin C from the nanoparticles was pH-dependent. Quick release took place in 0.1 M phosphate buffer solution (PBS, pH 7.4), while the release was slow in 0.1 M HCl. In addition, in vivo release rate in digestive tract of rainbow trout nearly showed the same trend as the in vitro one.

1. Introduction

Vitamin C is an indispensable nutrient required to retain the physiological process of human and different animals (Esposito, Cervellati, Menegatti, Nastruzzi, & Cortesi, 2002; Shiau & Hsu, 1999). Vitamin C is one of the most important antioxidants that may reduce the risk of cancer using various mechanisms (Esposito et al., 2002; Jacobs et al., 2001; Shklar & Schwartz, 1996; Zhang & Wakisaka, 1997). In addition, in humans and many animals, vitamin C is not synthesized in digestive tract due to the absence of enzyme L-gulonolactone oxidase that is responsible for the synthesis of vitamin C or ascorbic acid (Wilson, 1973), therefore they depend upon exogenous sources of vitamin C.

As a whole, the sustainability of vitamin C is low 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, Jauncey, & Roberts, 1987). Shiau and Hsu (1993) found that approximately 75% of the initial amount of supplemented vitamin C in shrimp feeds was lost during processing at ambient temperature. The utilization of more stable forms of vitamin C is therefore a crucial requirement for human and animal nutrition. The enhanced stability of vitamin C is a suitable option to realize its sustainability.

Encapsulation is a technique that has recently been utilized for shelf life extention of vitamin C in the form of microparticles. For example, different acrylic compounds were considered, namely Eudragit Rl L and RS. Spray drying has been used as preparation method of vitamin C/Eudragit microspheres (Esposito et al., 2002) where the particle size is in the micro range. This technique reduces the exposure of vitamin C to environmental factors such as temperature and oxygen.

In the present work, chitosan nanoparticles with different diameters have been used to encapsulate vitamin C in order to enhance its shelf life and to study the delivery system of the vitamin.

Chitosan is a biodegradable carbohydrate that has numerous applications and uses in various fields. Its superior characteristics have made it a favorite candidate to be employed in the nanotechnology field (Pillai, Paul, & Sharma, 2009).

2. Materials and methods

2.1. Materials

Chitosan with the deacetylation degree (DD) of 90% and molecular weight (Mw) of 65, 90, 110, 250 and 450 kDa was prepared according to a previous study (Abdou, Nagy, & Elsabee, 2008). The molecular weights were measured by the viscometric method while the DDs were evaluated using elemental analysis data (Abdou et al., 2008). Phosphate-buffered saline (PBS) tablets (pH 7.4), sodium tripolyphosphate (STPP) and vitamin C (sodium ascorbate) were purchased from Sigma (St. Louis, MO). Other reagents were of analytical grade and used without any further purification.

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doi:10.1016/j.foodchem.2010.11.086

2.2. Preparation of chitosan nanoparticles

Chitosan solutions of different molecular weights were prepared by dissolving chitosan in 1% (w/v) acetic acid solution until the solution was transparent. Sodium tripolyphosphate (0.5 mg/ml) was dissolved in deionized water. The chitosan solution was flush mixed with an equal volume of STPP solution and the formation of chitosan-STPP nanoparticles began spontaneously via the STPP ionic gelation mechanism. The nanoparticle suspensions were gently mixed for 60 min at room temperature before being used for further analysis.

2.3. Preparation of vitamin C nanoparticles

Chitosan-STPP/vitamin C nanoparticles were prepared via ionotropic gelation between the positively charged amino groups of chitosan-STPP and the vitamin C. Different masses of vitamin C were dissolved with chitosan-STPP solution having various Mws and then the mixture was subjected to mild magnetic stirring at room temperature for 1 h to affect crosslinking. The nanoparticles were isolated using ultracentrifugation at 2.18×10^4 g value. Then the supernatant was discarded and the precipitate was freeze-dried and stored at 4 °C until further analysis.

2.4. Physiochemical characteristics of chitosan-STPP/vitamin C nanoparticles

The particle size and zeta potential were determined using Zetasizer 3000HSA (Malvern Instrument, London, England). The morphology was observed under a scanning electron microscope (SEM, XL30, Philips, Amsterdam, Netherlands).

2.5. Determination of vitamin C encapsulation efficiency and nanoparticles yield

The encapsulation efficiency of the nanoparticles was analyzed using ultracentrifugation of the suspension at 4.44×10^4 g value. The precipitate was freeze-dried and weighted. The amount of vitamin C in the supernatant was determined by HPLC (HP 1100 Series, Waldbronn, Germany). In brief, 1 ml of supernatant were extracted with the same volumes of 4.5% (w/v) metaphosphoric acid solution and filtered through a 0.45 μm Acrodisc filter. An aliquot then was injected into the chromatographic column. The chromatographic system consisted of a quaternary pump, a vacuum degasser, a rheodyne 20 μl injection loop, a diode-array detector, and it was controlled through a HP Chemostation software. A Hypersil ODS column fitted with a Hypersil ODS guard column was utilized with a mobile phase of HPLC grade water with metaphosphoric acid to pH 2.2 at a flow rate of 0.5 ml/min. The detection was at 245 nm (Oruna-concha, Gonzalez-Castro, Lopez-Hernandea, & Simal-Lozano, 1998). The yield of the nanoparticles was dry weight of nanoparticle precipitate. Then the encapsulation efficiency (EE) was assessed using the following equation:

$$EE = [(\text{total vitamin C} - \text{free vitamin C})/\text{total vitamin C}] \times 100.$$

2.6. Shelf life assessment of vitamin C

Shelf life evaluation of vitamin C was determined using the application of vitamin C in encapsulated and non-encapsulated forms in rainbow trout (*Oncorhynchus mykiss*). The feed was stored for 20 days under good ventilation at room temperature (25 °C). After predetermined time, vitamin C content of the feed was analyzed. All measurements were performed in triplicate.

2.7. In vitro release test

Approximately 20 mg of powder nanoparticles were suspended in 5 ml of 0.1 M HCl or 0.1 M PBS and then incubated at 37 °C under mild stirring (120 rpm). At selected time intervals, (between 0.3 and 168 h) samples were ultracentrifuged. One millilitre of the supernatant was separated and the free vitamin C was determined like the above mentioned. All experiments were carried out in triplicate.

2.8. In vivo release test

Comparison of the in vivo delivery efficiency was conducted by inserting the nanoparticles in rainbow trout diet (*O. mykiss*) (200 g). At the predetermined time intervals, the stomach and intestine were isolated and pre-weighted samples were homogenized in 10% cold metaphosphoric acid. The homogenates were ultracentrifuged and the supernatants were analyzed on HPLC after being filtered through a 0.45 mm pore-size syringe filter (Sartorius, Gottingen, Germany). Then the vitamin C evaluation was determined like the in vitro experiment.

2.9. Statistical analysis

All experiments were carried out in triplicate and the results were expressed as mean ± SD. Analysis of variance was performed using SPSS statistical package program (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Preparation of chitosan-STPP nanoparticles

Chitosan ability of quick gelling on contact with polyanions (Scheme 1) relies on the formation of inter- and intramolecular crosslinking mediated by these polyanions.

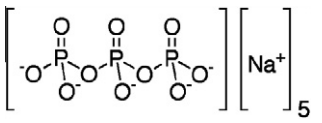
Nanoparticles are formed immediately upon mixing of STPP and chitosan solution as molecular linkages were formed between STPP phosphates and chitosan amino groups. Size and size distribution of chitosan-STPP nanoparticles depend largely on molecular weights of chitosan. (Gan, Wang, Cochrane, & MaCarron, 2005; Mao, Sun, & Kissel, 2009).

During the nanoparticles preparation, STPP is attracted to the amino groups in chitosan to create ionically crosslinked chitosan (Aral & Akbuğa, 1998; Sezer & Akbuğa, 1995; Yang & Hon, 2009). As soon as chitosan and STPP were blended together in dilute acetic acid, they made nanoparticles showing an overall positive surface charge.

3.2. Nanoparticles characterisation

The STPP/chitosan nanoparticles will interact with vitamin C through ionic and hydrogen bonding interactions.

Fig. 1. shows the effect of molecular weight of chitosan on the size of the nanoparticles. Fig. 1 illustrates the particle size distribution of the chitosan/vitamin C nanoparticles prepared using chitosan molecular weight of 450 kDa (350 nm). The particles prepared using 110 and 65 kDa chitosan, having diameters of 215 nm and



Scheme 1. Sodium tripolyphosphate (STPP).

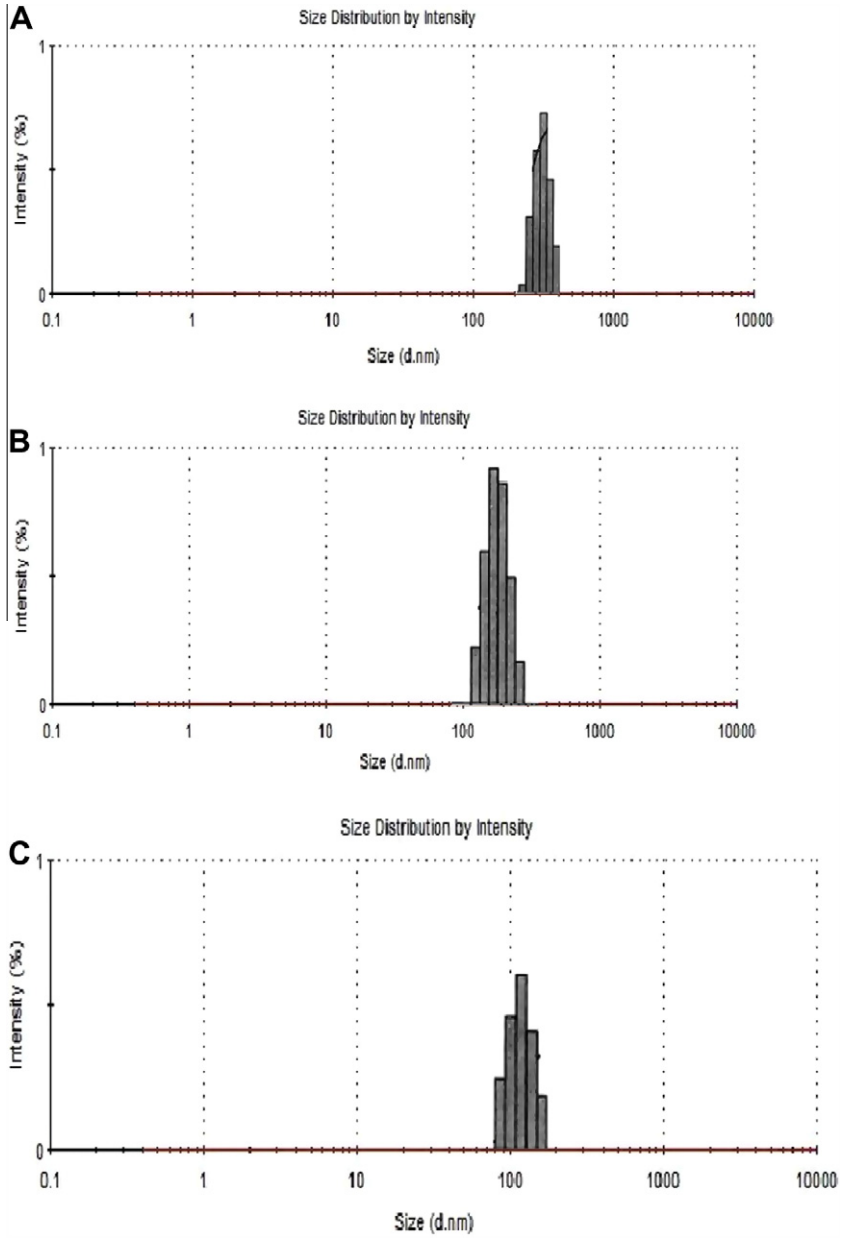


Fig. 1. Particle size distribution of chitosan/vitamin C nanoparticle with different Mw (A) 450, (B) 110 and (C) 65 kDa.

90 nm, respectively, showed similar morphology. Fig. 1 indicates that decreasing the molecular weight brings about a decrease of the diameter.

Fig. 2 shows the scanning electron microscopy (SEM) images of the chitosan/vitamin C nanoparticles. SEM micrographs attested the nanoscale size and spherical shape of the particles. As shown in Fig. 2, the lower molecular weight, the smaller the nanoparticles formed. Gan et al. (2005) illustrated that bovine serum albumin (BSA) incorporated into chitosan-STPP nanoparticles possessed a typical shape and smooth surface. Xu and Du (2003) and Janes, Calvo, and Alonso (2001) obtained similar results. In addition, they stated that the higher molecular weight of chitosan formed larger nanoparticles.

Table 1 presents the zeta potential values and average particle sizes of the prepared nanoparticles. The zeta potential of nanoparticles was positively charged and so it was presumed that chitosan/STPP nanoparticles surrounded the outer layer of the nanoparticles. These outcomes indicate that the formation of nanoparticles depended dramatically on the concentration of free amino groups, which enhanced the surface charges and zeta potentials of the nanoparticles and reinforced the electrostatic interaction between the nanoparticles and vitamin C, helping to maintain the spherical shape of the bead and reduce the particle size (Aral & Akbuğa, 1998; Sezer & Akbuğa, 1995). These data imply that chitosan of the appropriate Mw should be selected to achieve the desirable particle size because the efficiency of the cationically modified particles depends strongly on the size of the particles, which determines their cellular uptake (Mao et al., 2009).

Furthermore, as shown in Table 1, the nanoparticles yield increased as the Mw increases. Gan et al. (2005) demonstrated that there is a linear increase of size and nanoparticle yield with increasing chitosan Mw. Based on this view, high Mw increases the particle surface charge and so the crosslinking bond with the negatively charged particles (vitamin C) increases and ultimately should increase the nanoparticles yield (chitosan-STPP/vitamin C) (Yang & Hon, 2009).

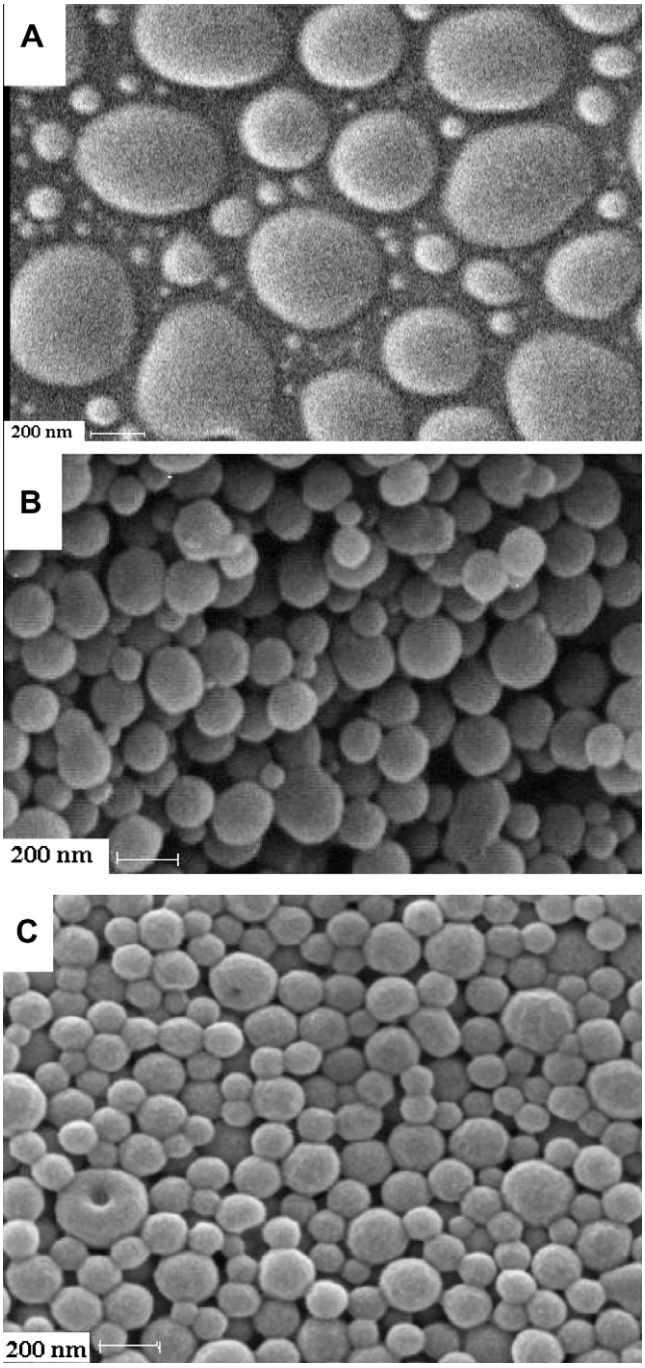


Fig. 2. SEM images of nanoparticles prepared using different Mw (A) 450, (B) 110 and (C) 65 kDa (50 kV × 30,000).

Table 1
Effect of chitosan Mw on the average particle size, zeta potential values and nanoparticle yield of chitosan/vitamin C nanoparticles.

Chitosan Mw (kDa)	Average particle size (nm)	Zeta potential values (Mv)	Nanoparticles yield (mg)
65	185.4 ± 2.1[a]	49.3 ± 1.6[a]	4.8 ± 0.1[a]
90	220.1 ± 1.8[b]	50.8 ± 2.5[b]	5.1 ± 0.1[b]
110	238.1 ± 3.2[c]	52.1 ± 4.3[c]	5.2 ± 0.2[b]
250	305.4 ± 4.2[d]	55.3 ± 2.1[d]	5.4 ± 0.1[c]
450	585.3 ± 3.6[e]	62.3 ± 1.5[e]	5.8 ± 0.2[d]

Values in the same column bearing different superscripts are significantly ($p < 0.05$) different.

3.3. Vitamin C encapsulation using chitosan nanoparticles

Vitamin C can be loaded into the nanoparticulate system in three mechanisms: (1) electrostatic interaction, (2) encapsulation, and (3) adsorption (Mao et al., 2009; Yang & Hon, 2009). Fig. 3 depicts the effect of Mw on vitamin C encapsulation efficiency. As shown in Fig. 3, the encapsulation efficiency of vitamin C increases up to 110 nm and then suddenly drops. In the present study, the best encapsulation of vitamin C was achieved at 110 nm.

Yang and Hon (2009) demonstrated that chitosan with a lower Mw contains shorter chitosan fragments, and thus making its free amino groups easier to protonate and, consequently, leading to greater adsorption of vitamin C through ionic interactions. On the other hand, some authors believe that higher Mw chitosan lead to higher encapsulation efficiency because longer chitosan chains could entrap more vitamins C. The positive effect of high chitosan Mw on vitamin C encapsulation was reasonable. The size of the nanoparticles may further affect their vitamin C encapsulation, as discussed in the previous sections. Therefore, the size increment of the chitosan-STPP/vitamin C nanoparticles might decrease vitamin C encapsulation efficiency because of the decrease of total surface area (Wang et al., 2009; Yoksan, Jirawutthiwongchai, & Arpo, 2010).

3.4. Shelf life assessment of vitamin C

As shown in Fig. 4, the shelf life of vitamin C encapsulated into chitosan nanoparticles is evidently higher than vitamin C without chitosan nanoparticle cover. Vitamin C is very sensitive to temper-

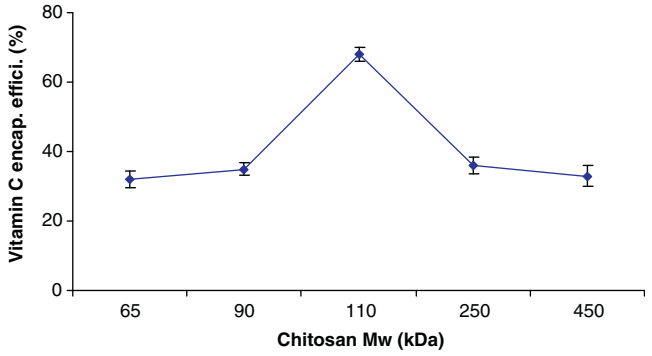


Fig. 3. Effects of chitosan Mw on the vitamin C encapsulation efficiency (chitosan concentration = 1 mg/mL, vitamin C concentration = 0.5 mg/mL).

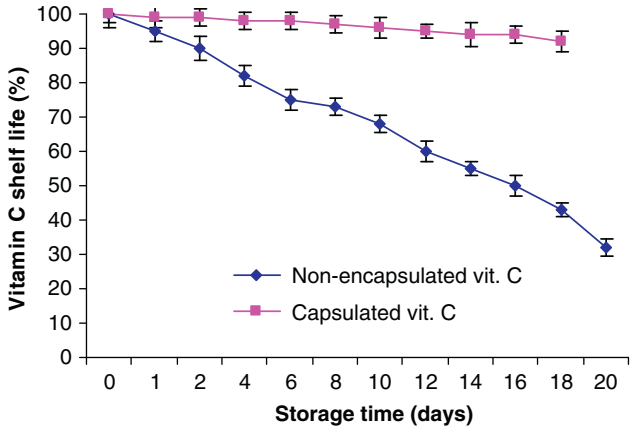


Fig. 4. Encapsulated and non-encapsulated vitamin C shelf life assessment in rainbow trout diet during 20 days storage time.

ature, oxygen, and light. In fish diet processing, vitamin C is spray added after the thermal processing due to sensitivity of vitamin C to high temperature. While vitamin C encapsulated with chitosan nanoparticles could be added into the diet before thermal processing since the nanoparticles surround the vitamin and thus preventing its direct contact with the environmental parameters such as light, heat and oxygen. In this way, the shelf life of vitamin C increases after encapsulation by the nanoparticles. Esposito et al. (2002) indicated that Eudragit microparticles encapsulating vitamin C showed a good morphology and size distribution permitting to propose them as candidate for the shelf life increment and delivery of vitamin C as associated therapy in the treatment of colorectal cancer by oral route.

3.5. In vitro release of vitamin C from chitosan nanoparticles

The release profiles of vitamin C from chitosan nanoparticles were investigated at 37 °C for 100 h. The media used were 0.1 M HCl and PBS as a simulation of stomach and intestine environment.

The amount of vitamin C released at different times was evaluated by HPLC. There were two stages of vitamin C release, depending on the release rate. As shown in Fig. 5A, the release of vitamin C in BSA from the chitosan-STPP/vitamin C nanoparticles was much faster than that from the nanoparticles in 0.1 M HCl in the same period. Only 30% of vitamin C was released in 0.1 M HCl, while more than 75% vitamin C was eluted out in PBS from the nanoparticles. This might be a result of the weakening of electrostatic interaction between the polyion complexes and the nanoparticles at the neutral pH.

As a whole, the release of vitamin C from the nanoparticles could take place in two ways: diffusion and erosion. In 0.1 M HCl, it is supposed that the diffusion process mostly controlled the process of vitamin C release. In PBS, in addition to the diffusion process, the ion exchange between polymer and release medium bring about the erosion of the nanoparticles and largely enhance the vitamin C release rate. Wang et al. (2009), Yang and Hon (2009) and Yoksan et al. (2010) demonstrated that in vitro release of the materials from the nanoparticles strongly depended on the medium pH. In addition, pH affected the electrostatic interaction between the materials and the nanoparticles. At neutral pH (intestine pH) the release of the material due to diffusion and erosion increases. On the other hand, in alkaline pH, mainly non-electrostatic interactions such as hydrogen bonding and hydrophobic bonding could be responsible for binding between chitosan and vitamin C (Mao et al., 2009). Consequently, because of the fragile nature of the non-electrostatic interactions, the release rate of vitamin C increases in neutral and alkaline pH.

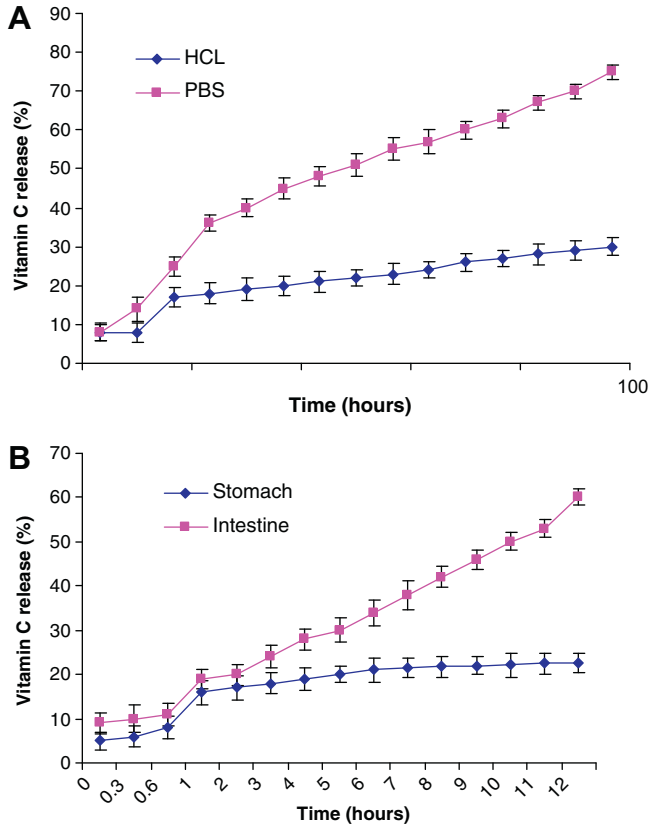


Fig. 5. (A) In vitro release of vitamin C from TPP-chitosan/vitamin C nanoparticles in 0.1 M HCl and in 0.1 M PBS, and (B) in vivo release of vitamin C from TPP-chitosan/vitamin C nanoparticles in the stomach and intestine of rainbow trout (*O. mykiss*) (chitosan Mw = 90 kDa, $n = 3$).

3.6. In vivo release of vitamin C from chitosan nanoparticles

Fig. 5B depicts the in vivo release of vitamin C from the nanoparticles. The results are in accordance with the in vitro outcomes and, thus, confirm them. Unlike in vitro release time, after 12 h, there was no observed vitamin C in the stomach and the intestine due to the high passage time of feeds in the digestive tract of rainbow trout. Of course, the passage time in rainbow trout was suitable due to the mucoadhesive characteristic of chitosan within the rainbow trout digestive tract (Dorkoosh, Verhoef, Tehrani, Borchard, & Junginge, 2003). During the residence time, the release rates in stomach and intestine have shown the same trend as in vitro release rate. In stomach of rainbow trout due to high acid content, the release rate was low because electrostatic interaction in acid environment between STPP-chitosan nanoparticles and vitamin C was strong and the release of vitamin C from the nanoparticles only took place by diffusion (Mao et al., 2009; Wang et al., 2009; Yang et al., 2009). On the other hand, in intestine, the accelerated release of vitamin C from the nanoparticles was more likely due to the reduced electrostatic interactions between the polyion complexes and the nanoparticles. In addition, due to the disintegration of the polyions matrix in the nanoparticles, the ion exchange between polymer and release medium cause the erosion of the nanoparticles and largely increases the release rate of vitamin C (Gan et al., 2005).

With regard to the above mentioned, vitamin C encapsulated with chitosan nanoparticles was a good option to increase the shelf life and delivery of vitamin C over the time (one week) that it was much important due to low stability of vitamin C to light, temperature and oxygen. Furthermore, the in vivo results demonstrated that the encapsulation of vitamin C was desirable barrier to maintain it from enzymes and acid hydrolysis in the stomach and intestine. Ultimately, the maintenance of vitamin C structure and functionality within digestive tract of rainbow trout using the STPP-chitosan nanoparticles is a novel perspective to overcome vitamin C susceptibility in digestive tract.

4. Conclusions

Novel STPP-chitosan/vitamin C nanoparticles system successfully increased the shelf life and delivery of vitamin C using ionotropic gelation method. Low Mw chitosan favoured higher vitamin C encapsulation efficiency. The release of vitamin C from nanoparticles was pH-dependent. Faster release was observed in PBS compared with that at low pH. Furthermore, in vivo release of vitamin C showed that the chitosan nanoparticles increased the residence time of vitamin C in rainbow trout digestive tract. The results showed that the release time was by 12 h as compared

with ordinary state that was 3–4 h, due to mucoadhesive nature of chitosan. The release rate of vitamin C in the stomach and intestine of rainbow trout showed the same trend as in vitro one, though to lesser extent. The results demonstrated that the STPP-chitosan/vitamin C nanoparticles have a promising potential to increase shelf life and delivery of vitamin C in biological systems.

Acknowledgments

The authors are grateful to Tehran University for fiscal support. In addition, the authors are much grateful to Mr. Khatir for editing the manuscript for its English.

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