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Encapsulation of Vitamin C by A Double-Layer Zein/Chitosan Structure with Improved Stability and Controlled Release

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Abstract

Encapsulation structures are of interest for drug delivery and controlled release purposes in the food, pharmaceutical, and cosmetics industries. In this study, a double-layer zein/chitosan structure was utilized to encapsulate vitamin C (VC). VC was first encapsulated by chitosan (CS) with the cross-linker sodium tripolyphosphate (STPP). Then zein was added to coat the formed VC-encapsulated CS nanoparticles. The formed VC-encapsulated zein/CS microspheres had a good sphericity with particle size ranging from 720 to 1100 nm. VC was greatly protected from degradation by the double-layer coating and only 5% of VC was oxidized after 10-daystorage at room temperature. Compared to the nanoparticles made by CS only, the double-layer structure had a better performance on retarding the release of VC in simulated gastric fluid (SGF, pH 1.2) and achieving controlled release in simulated intestinal fluid (SIF, pH 7.4), which provided a method for further utilization of nutrients in human body.

Keywords: Vitamin C; Chitosan; Zein; Encapsulation; Controlled release

Introduction

Vitamin C (ascorbic acid, VC), a weak sugar acid, is an essential nutrient in human body which has been proved to be associated with many physiological processes of humans and animals [1,2]. It is one of the most important antioxidants in human tissues, with a variety of biological functions, such as immune system enhancement [3], oncogenic micro-organism inhabitation, and free radical damage prevention [4]. Furthermore, these functions of VC can be correlated with its potential to lower the incidence of cancers [5,2]. However, VC cannot be synthesized in human body because of the lack of enzyme L-gulonolactone oxidase [6]. Daily VC intake from exogenous sources is required to guarantee the regular physiological processes of human body. The challenges encountered in the VC supplementation rise from the instability of VC in normal food processing and during food intake of human body. VC is soluble in water and very sensitive to oxygen, light, and heat. Decomposition would easily occur in neutral or alkaline conditions [7]. In order to conquer these problems as well as lengthen the shelf life of VC and enhance its effective absorption in human body, different techniques and formulations, including micro-encapsulation spray drying, spray cooling, and liposomes, are investigated. For example, VC was encapsulated into liposomes and its shelf life was longer than VC in free solution [8]. In recent years, gum arabic was used as shell material to encapsulate VC by spray drying and the result showed that the encapsulated VC was as stable as the crystallized VC under normal conditions [9]. Later, VC-loaded microcapsules were formed by different methods, such as melt dispersion, solvent evaporation, and thermal phase separation. Those formed capsules could successfully prevent VC from color change, improve its taste, and decrease its release rate [10].

Micro-encapsulation is a process to incorporate solid, liquid, or gaseous ingredients in micro scale capsules, which can isolate the core material from the surroundings to enhance the stability and retard evaporation. It also contributes to the controlled release of therapeutical ingredients in human body [11,12]. This technique is valued in food and pharmaceutical industries in recent years. A wide spectrum of coating materials has been developed. For example, chitosan, a hydrophilic polysaccharide of low toxicity, has been proved to be used in the microencapsulation of VC for its oral delivery with the assistance of sodium tripolyphosphate (STPP), a non-toxic cross-linker with quick gelling capability [13]. However, fast release profile was observed from the VC-loaded CS nanoparticles in Phosphate Buffered Saline [13-15]. Therefore, a secondary coating of zein on CS is proposed to get a better protection of VC.

Zein, a prolamine protein stored in corn endosperm, is well applied in food and pharmaceutical industries because of its good biocompatibility and bioavailability. Zein can self-assemble to form biobased films, hydrophobic surfaces, and encapsulations, and has potential applications in food packaging, electronic devices, and for oral administration enhancement [16]. Zein is an amphiphilic protein which can be dissolved in 75% ethanol aqueous solution [17]. After solvent evaporation, it can self-assemble to form microsphere. Studies have proved that there was a conformational transition from α-helix to β-sheet happening during the evaporation-induced self-assembly (EISA) process while EISA is a technique used to promote self-organization of nanostructures [18,17]. EISA is a process that involves binary or tertiary solvents. With the preferential evaporation of one of the solvents, the polarity of the solution changes, which drives the self-assembly of solutes. Because of its self-assembly ability, zein has been extensively studied to encapsulate bioactive compounds, such as essential oil [19], flax oil [20], vitamin D3 [21],

α-tocopherol [22], and citral and lime [23]. Comparing with other wall materials, zein coating greatly improves the controlled release function of the encapsulation, including both the release delay and the prevention from stomach solution. However, the core material selection and the size of delivery particles are still the limitations for its wide applications.

The core materials selected for zein encapsulations are generally hydrophobic or amphiphilic. This is related to the intrinsic tendency of zein to aggregate around these materials during EISA. The formation of self-assembled spheres could be subsequently triggered after the aggregation of zein around the core materials. However, this process is less applicable for highly hydrophilic substance, such as heparin [24], 5-fluorouraci [25], and pDNA [26]. In spite of some reported experiments about the direct preparations of the above non-hydrophobic encapsulated zein micro/nanoparticles, the morphology of the products was not satisfactory, which inevitably affected the encapsulation efficiency and drug delivery. Moreover, in these studies of micro/nano encapsulation, coacervation method is widely adopted to access smaller size. The micro/nanoparticles are obtained by desolvation of zein through the sudden addition of an aqueous solution while vigorous homogenizing or stirring is performed. However, the disadvantage has been pointed out that it may cause the loss of labile encapsulated ingredients in such high-energy method [22].

The objective of this work is to use chitosan and zein to prepare a double layer encapsulation of VC. SEM was used to characterize the morphology of the particle, and ZETASIZER was used to measure the particle size and size distribution of the encapsulations. HPLC and electro spray ionization mass spectrometry (ESI-MS) was used to measure the VC amount in the encapsulation and investigate the stability of encapsulated VC. And in vitro gastrointestinal studies were conducted to study the release profile of zein-CS encapsulation of VC with a comparison of the encapsulation made by CS only.

Materials and Methods

Materials

VC (L-Ascorbic acid, 99.7% purity) and Sodium tripolyphosphate (STPP) were purchased from Tianjin Damao Chemical Co. Ltd. (Tianjin, China). Chitosan (CS) with the deacetylation degree of 80% and molecular weight of 65 kDa, pepsin (from porcine gastric mucosa) and pancreatin (from porcine pancreas) were obtained from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Zein was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Ethanol (96% v/v) was from Guangdong Guanghua Sci-Ttch Co. Ltd (Guangzhou, China). All other reagents were of analytical grade. All chemicals were used as received.

Preparation of VC-encapsulated CS nanoparticles

CS (2.0 mg/ml) was dissolved in 1% (w/v) acetic acid solution under continuous stirring for 20 min to get homogeneous solution. VC aqueous solution (1.0 mg/ml) was added slowly into CS acetic acid solution and stirred at 1000 rpm for 30 min at room temperature. STPP solution (0.5 mg/ml) was then added drop wise to the above mixture solution under stirring at 1000 rpm for another 30 min to form VC-encapsulated CS nanoparticle suspension. The solution container was covered by aluminum foil all the time to avoid VC degradation. The VC-encapsulated CS nanoparticles were obtained by ultracentrifugation (2.18×10^4 g) and freeze-drying.

Preparation of VC-encapsulated zein/CS microspheres

To prepare VC-encapsulated zein/CS microspheres, a certain amount of ethanol was added to the prepared VC-encapsulated CS nanoparticle suspension to make a strong alcohol solution with ethanol: water 70:30 (v/v). Then 1, 1.5, 2, 2.5, and 3 ml zein solution (10 mg/ml dissolved in 70% ethanol-water) were then added drop wise under continuous stirring, respectively. The mixed solutions were stirred at 1000 rpm for 30 min and poured on the evaporating dishes. The VC-encapsulated zein/CS microsphere powder was gained through EISA at room temperature (25°C). The prepared samples were stored at 4°C until further analysis.

Particle size evaluation and morphological observation

The particle size of VC-encapsulated CS nanoparticles was determined using Malvern ZETASIZER 3000HSA (Malvern Instrument, London, England). The particle size of VC-encapsulated zein/CS microspheres was estimated through scanning electron microscope (SEM). Image J software was applied for statistical treatment for SEM images of the selected VC-encapsulated zein/CS microspheres.

The sample particles were resuspended in deionized water and cast-dried on the foil under room temperature overnight. The foil was cut into small slices to adhere to carbon tapes. The whole tapes were then gold coated (300Å) with an Edwards S150B sputter coater to help improve electrical conductivity of sample surface. SEM images were obtained with a JEOL JSM-6490 SEM (Tokyo, Japan). The size of the microspheres was measured in a random area under microscopic field at certain resolution. A total of 6 random areas were selected in each specimen to obtain the average diameter and the data was processed by Image J. SEM was also used to analyze the morphology of VC-encapsulated zein/CS microspheres.

Encapsulation efficiency

The encapsulation efficiency (EE) of VC-encapsulated CS nanoparticles was analyzed by measuring the amount of free VC in the supernatant obtained by ultracentrifugation of CS nanoparticle suspension and comparing it with the total amount of VC calculated by the solid mass ratio. The VC loading efficiency (LE) was also calculated. The amount of released VC was determined by RP-HPLC analysis with UV detection at 245 nm [13]. Separation was performed on an ODS column (LiChrospher RP-18 5u, 250 × 4.6 mm). The unitized mobile phase was methanol, and the flow rate was set at 0.5 ml/min. The drug EE and LE were calculated using the equations 1and 2:

$$EE = \frac{total\,VC - free\,VC}{total\,VC} \times 100 \quad \text{.....(1)}$$

$$LE = \frac{mass\ of\ VC\ in\ particles\,(mg)}{mass\ of\ particles\,(g)} \quad \text{.....(2)}$$

ESI-MS analysis

The presence and stability of VC in zein/CS microspheres were examined using ESI-MS. 1 mg of prepared VC-encapsulated zein/CS microspheres (10 days after preparation and stored at room temperature, 25°C) were first suspended in deionized water. The solution was then centrifuged at 10,000 g for 15 minutes at 4°C and then the supernatant liquid was discarded to remove any residual VC on the surface of the microspheres. The microsphere left at the bottom was resuspended in 200 μL of methanol: water 80:20 (v/v) and sonicated for 5 min. The sample was then centrifuged for 1 min for sedimentation of the solid powder residue. The supernatant was directly analyzed by ESI-MS operated in negative ionization mode.

Release test in simulated gastrointestinal fluids

The release tests of VC encapsulations were carried out in simulated gastric fluid (SGF, pH1.2, with pepsin) and simulated intestinal fluid (SIF, pH7.4, with pancreatin) with continuous stirring at constant 37℃. 10 mg of microsphere powder was suspended in 5 ml of SGF/SIF in dialysis membrane bag with molecular weight cut off at 10 kDa. Then the dialysis membrane bag was placed in 100 ml SGF/SIF without pepsin/

pancereatin. The whole system was kept at constant 37°C with continuous stirring. At selected time intervals, 1 ml solution outside the dialysis membrane bag was collected and 1 ml fresh solution was supplemented to the release system. The 1 ml solution taken from the release system was first filtered through a 0.45 μL Acrodisc filter to remove the suspended protein aggregates and used to determine the amount of released VC directly by reverse phase HPLC. The HPLC conditions were the same with that described in section 2.5.

Statistical analysis

All experiments were carried out in triplicate and the data was expressed as mean ± standard deviation (SD). The analysis of variance (ANOVA) with Turkey tests was used to analyze the differences between groups. The significance level of the test was set to be 0.05.

Results and Discussion

Particle size and morphology

The average diameters of different particles were listed in table 1, and the SEM images of selected microspheres are shown in figure 1. The VC-encapsulated CS nanoparticles in the preparation conditions had a diameter of about 240 nm. Further encapsulation of zein increased the particle size. CS has the ability to gel with polyamines like STPP and form micro globules, the process is known as ionotropic gelation. In dilute acidic solution (1% acetic acid, pH = 2.8), CS (pKa = 6.3) is protonated and its glucosamine units convert to $R\text{-}NH_3^+$. Then the counter-anion STPP added could quickly form intermolecular cross linking with CS and precipitate it in the form of nanoparticle. Alishsahi and coworkers [13] have studied the encapsulation of VC by mixing the VC with CS-STPP. The formation of VC-encapsulated zein/CS microspheres can be explained by the different behaviors of CS and zein during solvent evaporation. The solubility and elasticity of CS gel in ethanol solution largely depended on the polarity of the solution. Evidence showed that, when the concentration of ethanol was larger than 50%, the solubility of CS gel decreased to almost zero [27]. It was considered that there was a contrast phase transition of zein and CS gel during the solvent evaporation. Zein self-assembled into regular spherical shape, while the chitosan gel maintained its elasticity in its poor soluble state. A solidified zein shell wrapped the chitosan in lower ethanol solution before the gel elasticity decreased. In this way, the VC could be well encapsulated inside the particles.

The particle sizes of the formed zein/CS microspheres were not linear with the zein concentrations. The zein/CS mass ratio of 2:1 produced the smallest particle size (720 nm). SEM images (Figure 1) also shows that the microspheres with zein/CS mass ratio of 1.5:1, 2:1 and 2.5:1 had better spherical integrity than others. The similar trends of particle deformation were observed at both low and high ratios of zein/CS. At low zein concentration (zein/CS mass ration<1.5:1), the particles merged together to form large agglomerates. This was because the amount of zein was not enough to form a fully covered shell on CS-STPP nanoparticles, then the half encapsulated particle would be attracted by each other by hydrophobic interactions when the solvent polarity increased, which also accounted

Sample	VC-CS	VC-zein/CS (mas ratio 1.5)	VC-zein/CS (mass ratio 2.0)	VC-zein/CS (mass ratio 2.5)
Size (nm)	240 ± 30	880 ± 130	720 ± 90	1100 ± 210

Table 1: The average particle sizes of different particles

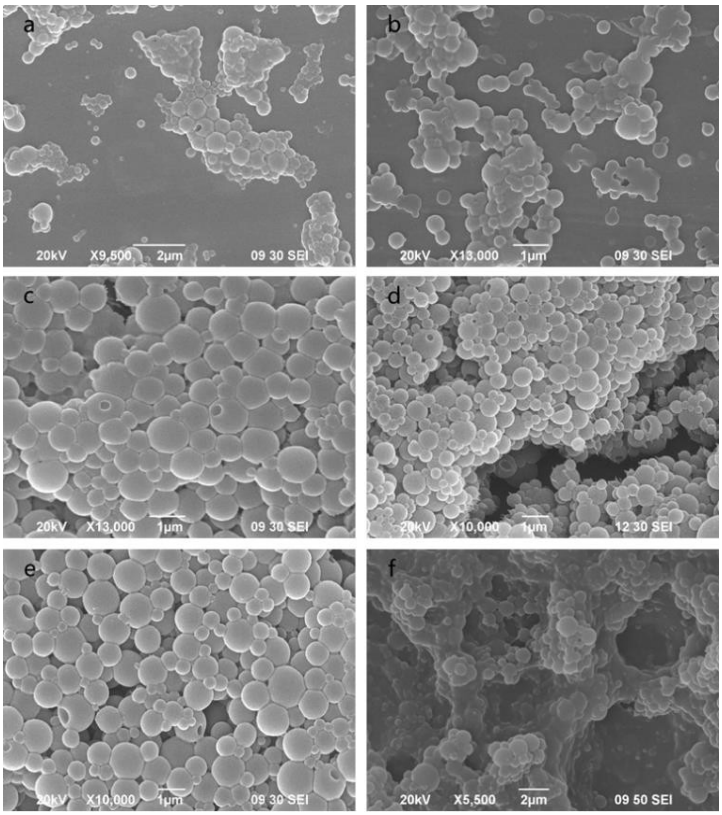

Figure 1: SEM images of VC-encapsulated zein/CS microspheres with different zein/CS mass ratio of (a) 0.5:1, (b) 1:1, (c) 1.5:1, (d) 2:1, (e) 2.5:1, and (f) 3:1.

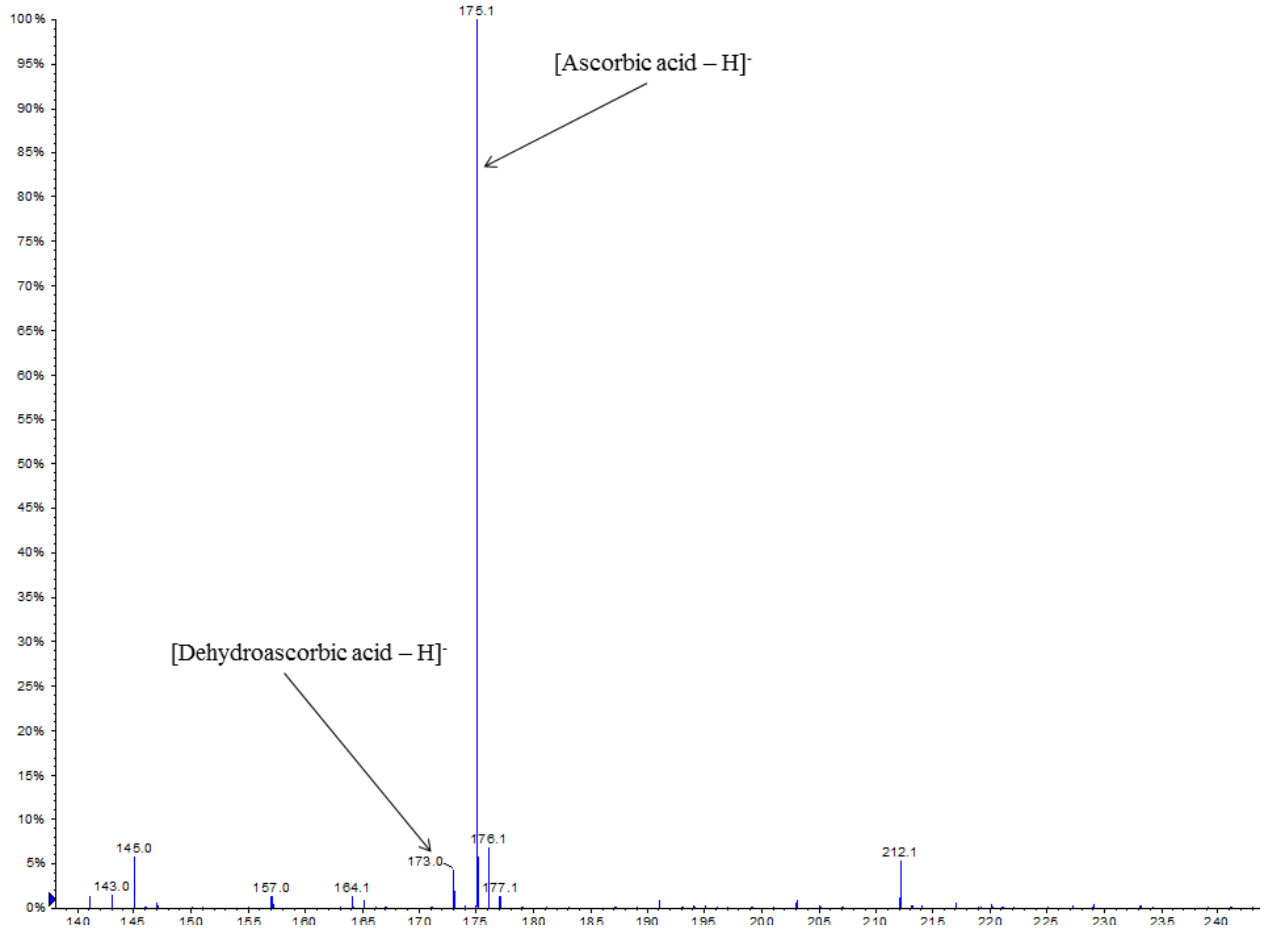

Figure 2: Negative ion ESI-MS spectrum for the VC-encapsulated zein/CS micro spheres.

for the wider size distribution. With increased zein concentration, the sphericity of particle increased with homogeneous size distribution. It was because a sufficient amount of polymer was provided for the formation of the shell structure on each CS-STPP nanoparticle. However, when the zein/CS mass ratio reached 3:1, the threshold of film formation achieved. The dispersed sphericity was caused by the fusion of zein surrounding the CS nanoparticles and excess zein in the solution matrix [17]. It indicated that the particles, which showed the most balanced tendency of particle merging and film extension, had the smallest particle size and largest particle density.

Stability of VC loaded in zein/CS mircrospheres

The sample of VC-encapsulated zein/CS microsphere (prepared with zein/CS mass ratio of 2:1) was examined by ESI-MS at room temperature after 10-day storage and the spectrum is shown in figure 2. The ESI-MS spectrum shows a predominant peak corresponding to VC (m/z 175), indicating that VC was the major chemical component of the sample. Besides the predominate peak, a small peak corresponding to dehydroascorbic acid (m/z 173), which was an oxidation resultant from ascorbic acid, was also detected and the intensity of this peak was about 5% of that of the peak of VC. The identification of the peak at m/z 173 was further confirmed by tandem mass spectrometric analysis (MS/MS) (Figure 3). So the stability test showed that, after 10-day storage, at least 95% of the encapsulated VC was well protected from oxidation. The 5% of dehydroascorbic acid, which was the oxidative resultant of VC, was also possible to be produced during the ESI-MS test. The above results showed that our encapsulation structure preformed a great protection to the VC inside.

Effect of different formations on *in vitro* release of VC

The release percentage of VC from the microspheres was calculated as the mass ratio of the VC detected in the release medium to that of the encapsulated VC. The amount of the encapsulated VC could be calculated in the way of the amount of the added VC times EE. As calculated according to section 2.5, EE and LE was 51.9% and 51.2 mg of VC per gram of microspheres, respectively. Figure 4 shows the in vitro release profiles of VC from CS nanoparticles (with 0.87 ± 0.12% moisture content) and zein/CS microspheres (prepared with zein/CS mass ratio of 2:1) in SGF at 37 for 420 min. A rapid burst of VC from CS nanoparticles was observed in the first 60 min, approximately 17% of VC being released. The release rate then decreased to 0 and a final accumulative amount of 20% was achieved at 300 min. In comparison, the release rate was much lower for zein/CS microspheres within the first 240 min, with only about 5% VC being eluted from the microspheres, and the release rate increased thereafter. In addition, the total released VC from CS nanoparticles at 420 min was nearly 26%, more than two times of that from zein/CS microspheres. The change of the release rate could be explained by the release mechanisms. The formation of VC-encapsulated CS nanoparticles was driven by the ionic interactions between the anionic counterion of VC and the amino groups of CS [14]. And VC molecules distributed evenly in the formed nanoparticles. Hence, for VC-encapsulated CS nanoparticles, the initial rapid release was attributed to the easy diffusion of VC through the cross-

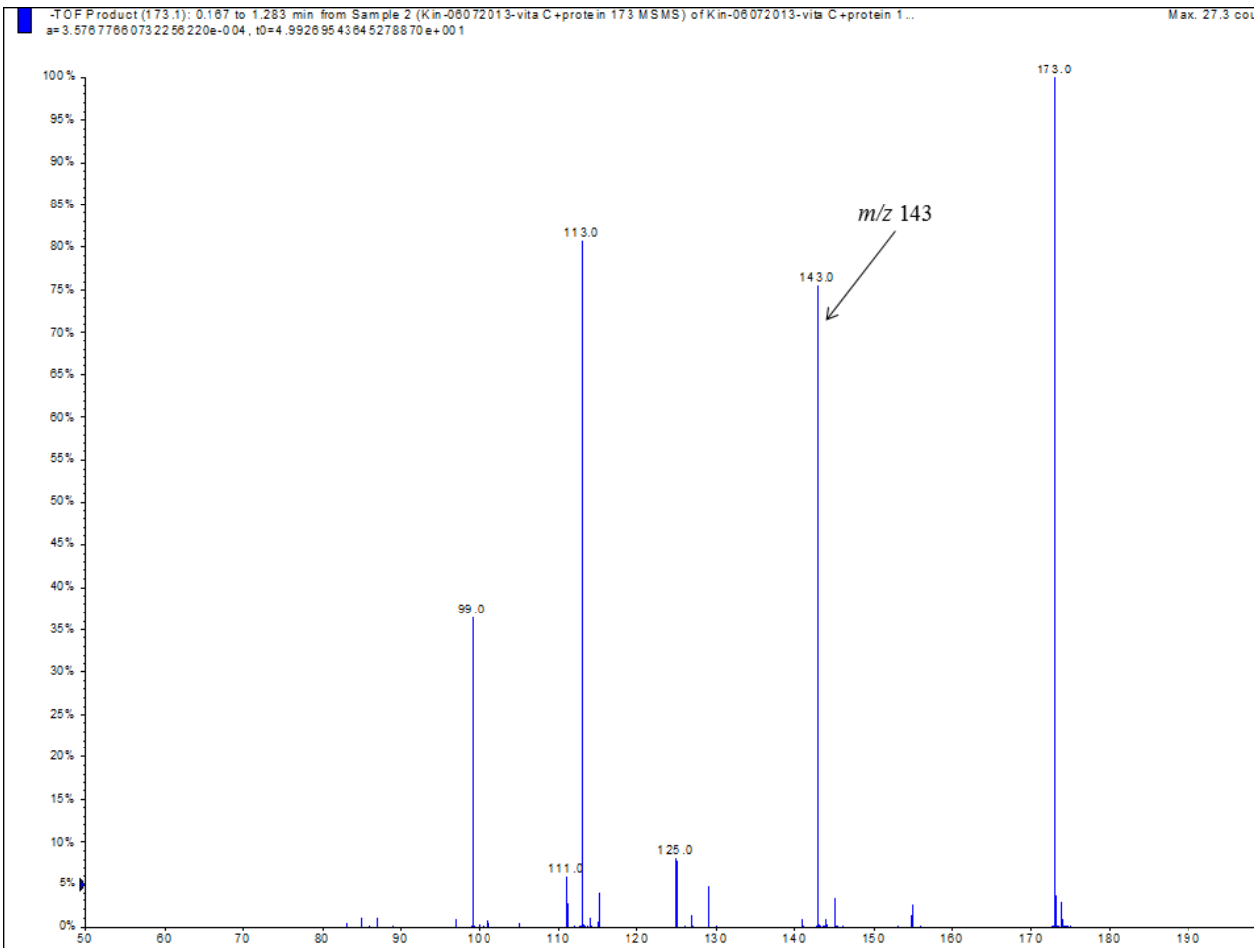

Figure 3: Negative ion ESI-MS/MS spectrum for the m/z 173 ion

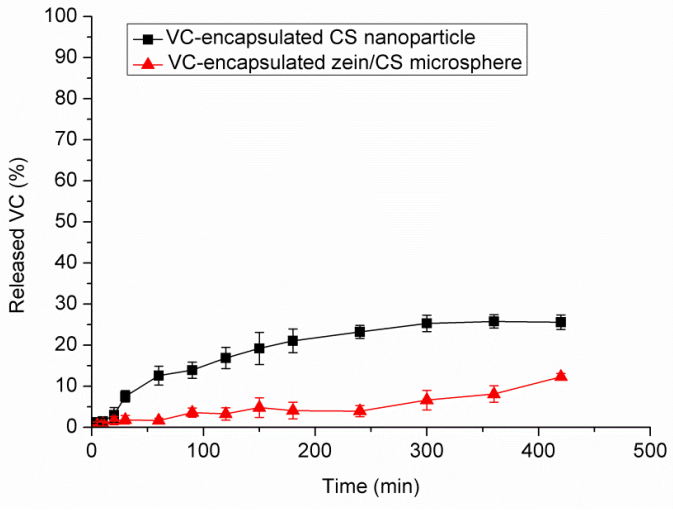

Figure 4: The in vitro release curves of VC from VC-encapsulated CS nanoparticles and VC-encapsulated zein/CS microspheres in SGF, respectively

linked polymer matrix to the particle surface [28]. And the gradually decreased rate in the later stage was mainly attributed to the degradation of CS, which cost more time. Furthermore, in acidic environment, the CS and STPP were strongly associated and thus prevented the matrix from degradation. On the other hand, for VC-encapsulated zein/CS microspheres, owing to the well-sealed zein shell, the slow degradation of zein played a principal role and thus an extremely low releasing rate was observed in the first 60 min. The later increased release rate of VC from zein/CS microspheres could be attributed to the change of release mechanism. A considerable extent degradation of zein shell was achieved after four hours of digestion and the CS core was exposed. Since then, the diffusion of VC molecules began to appear, presenting a trend of increased release.

Release of VC from zein/CS microspheres in different simulated fluids

The cumulative release of VC from zein/CS microspheres was investigated in SGF and SIF, respectively. The release profile is showed in figure 5. A burst release of VC was observed in SIF in the first 10 min with a releasing amount of 20%. After that, the release rate gradually decreased, finally accounting for 65% of total release, which was much higher than the total 26% in SGF. The obvious difference in release profiles between the above two were due to the effects of different digestive enzymes on zein. According to Hutrado et al. [29], pepsin in gastric fluid can only digest α-zein whereas pancreatin in intestine can digest both the α-zein and the α-zein dimers, resulting in a faster decomposition of zein spheres. Figure 5 shows that the outer zein coating could be digested within 10 min and VC was quickly eluted from the exposed CS particle surface. However, since the limited ability of pepsin to digest zein, a relatively lower release rate was observed at the early stage in SGF. Moreover, the overall increased

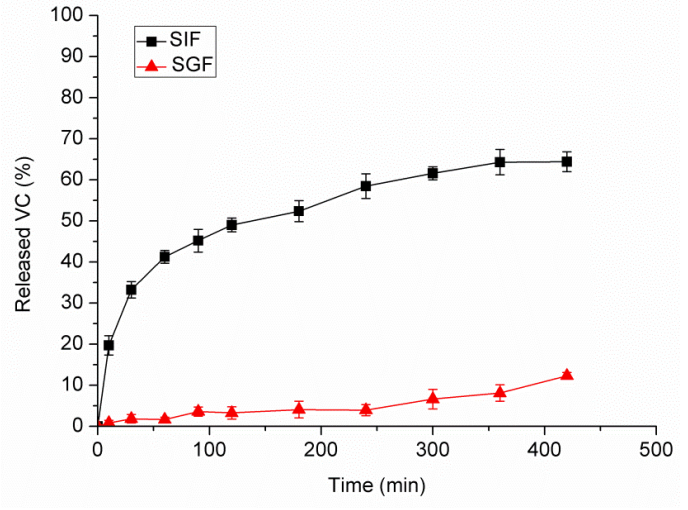

Figure 5: The in vitro release curves of VC from VC-encapsulated zein/CS microspheres in SGF and SIF, respectively

release rate in SIF was also affected by the reduced electrostatic interaction inside CS nanoparticle due to the high pH value. The erosion of zein and CS both caused the higher eluting level of VC in intestine.

Conclusion

VC was encapsulated by a double-layer structure of zein and CS. The ratio between zein and CS was studied for the formation and optimization of the double-layer encapsulation and SEM was used for the structure characterization. The ESI-MS results showed that the VC was successfully encapsulated and protected by zein and chitosan. The controlled release study was carried out using simulated gastrointestinal fluids. The double encapsulation of VC by adding an extra zein shell to the gelled VC-encapsulated CS nanoparticle successfully protected the drug from degradation, delayed the release in the stomach, and promoted the release in the intestine. The result indicated that this double-layer encapsulation is potential for the applications of encapsulations for other polar drugs to achieve controlled release.

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