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Preparation of chitosan–hyaluronate double-walled microspheres by emulsification-coacervation method

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Abstract Chitosan (CHS)−hyaluronate (HA) double-walled microspheres were prepared by emulsification-coacervation method. Tripolyphosphate (TPP) acted as ion crosslinker. The effects of oil/water volume ratio, surfactant, solution pH, TPP concentration, HA concentration, and emulsification time on microspheres fabrication and morphology were examined by Zeta (ζ) potential, Scanning electron microscopy (SEM) and Fourier-transform infrared spectrometry (FT-IR). It was found that TPP concentration, solution pH, surfactant and emulsification time were crucial factors for microspheres fabrication. Spherical microspheres with smooth surface were formed when TPP concentration was 8% or higher. The optimal pH for microspheres formation ranged from 6.0 to 7.0. As for surfactant, the microspheres obtained when span80 was applied alone were shapelier compared with those obtained when both span80 and tween80 were applied. With insufficient emulsification time, vacuous microcapsules, but not compact microspheres were formed. In addition, oil/water volume ratio and HA concentration also affected the microspheres morphology, but less importantly.

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Introduction

Among the various ways of achieving long-term drug delivery, polymeric microspheres have been effectively used for many years [1, 2] because of their biocompatibility, high bioavailability, ability to encapsulate a variety of drugs, and sustained drug release characteristics [3]. However, conventional single-layered microsphere has several shortcomings, such as high initial burst release effect and unsatisfied release mode [4, 5]. To overcome these problems, double-walled microsphere was introduced. There were accumulated evidences that it was an excellent method to apply drugs. It had higher drug encapsulation efficiency compared with conventional microspheres [6] and could reduce the initial burst release effect [7–9]. Furthermore, the release profile for microspheres with drug in the inner core was very close to a zero-order release profile and this was more pronounced at higher loading where the initial burst effect tends to be larger [10]. All those advantages were especially important for protein drugs, and could make the use of protein drugs more safely.

Because of its mild characteristics, physical crosslinking or coacervation technology has been applied in proteins drugs delivery systems. Emulsification-coacervation is a method based on ion crosslinking and coacervation; it is usually used to prepare double-walled microspheres. Emulsification-coacervation method has been used in the preparation of microspheres loaded with small-molecule substrates, DNA and proteins [11, 12]. However, to the best of our knowledge, for this method, the detailed influences of different conditions on microspheres formation are unreported.

In our study, sodium hyaluronate (HA) and chitosan (CHS) were selected after careful consideration of their biochemical characteristics: HA is a component of extracellular matrix (ECM); it has excellent biocompatibility,

viscoelasticity and hygroscopicity [13–15]. Chitosan is biodegradable, biocompatible, and nontoxic [12, 16, 17]. Both HA and CHS have been widely used in drug delivery systems. However, few studies have examined them simultaneous and only one study describing double-walled microsphere formed with CHS and HA has been reported [18]. In this particular study, the microsphere size was big (average diameter was 1550 μm) and thus limited its usage as a drug delivery system.

The aims of current study were: (1) to prepare double-walled CHS–HA microsphere as a protein drugs carrier using emulsification-coacervation method; (2) to investigate the effects of several factors on microspheres preparation: oil/water volume ratio, surfactant, solution pH, TPP concentration, HA concentration, and emulsification time.

Materials and methods

Materials

CHS (M_w 75,000 Da, 85% deacetylation) was provided by Rihuan Co. (Zhejiang, China). HA (M_w 2,000,000 Da) was supplied by Freda Co. (Shandong, China). Pentasodium tripolyphosphate (TPP) was purchased from Sigma Chemical Co. (U.S.A). All other chemicals were of analytical grade.

Preparation of CHS–HA microspheres

Chitosan was purified to remove impurities and low molecular weight substances. 5 g chitosan was dissolved in 500 mL of 2% acetic acid, filtered and adjusted to neutral pH with 0.5 M NaOH. The precipitant was collected and centrifuged at 3500 rpm for 5 min, then resuspended in deionized water. The washing step was repeated three times, and chitosan was lyophilized for storage. To prepare for microsphere formation, purified chitosan was dissolved in acetic acid solution (3% w/v) and adjusted to pH 3.5.

CHS–HA microspheres were prepared by the emulsification-coacervation method. 2 mL chitosan solution (pH 3.5, as inner water phase) was added dropwise into 50 mL paraffin liquid (oil phase) containing 1% (v/v) span80 as emulsifier. The mixture was stirred at 600 rpm using a homogenizer (IKA, RW 20 DZM.n, Germany) and became steady after 15 min, then a mixture solution of TPP and HA containing tween80 or not (as outer water phase) was added into the oil phase dropwise, and emulsified by stirring at 600 rpm. After the crosslinking time, the mixture was centrifugated at 15,000 rpm for 10 min at 4 °C. Then, the microspheres were washed twice in deionized water containing tween80 by centrifugating at 4,000 rpm for 5 min and were freeze-dried.

The experiments were conducted at different oil/water volume ratio (1:0.2, 1:0.4, 1:0.6, 1:0.8, 1:1.0, 1:1.2), solution pH value (4.0, 5.0, 6.0, 7.0, 8.0), TPP concentration (1%, 3%, 5%, 8%, 10%), HA concentration (0.025%, 0.05%, 0.1%, 0.2%, 0.4%) and emulsification time (0.5 h, 1 h, 2 h, 3 h) to assess their effects on microspheres formation.

Characterization of CHS–HA microspheres

Surface and interior morphology

Morphology of the microspheres (transversal sections obtained by cryofracture in liquid nitrogen) was observed using scanning electron microscopy (SEM): samples were sputter coated with gold using a JEOL JFC-100 ion sputter device, and then examined under a JEOL JSM-6700F scanning electron microscope (Japan).

Surface charge

Measurement of zeta (ζ) potential was performed with a zeta potential analyzer (Zetasizer 3000HS, Malvern, UK): The dried microsphere samples were suspended in deionized water (pH 7) and sonicated before measurement. The obtained homogeneous suspensions were used to determine zeta (ζ) potential. Each measurement was performed in triplicate.

Size analysis

Particle size was measured with Dynamic light scattering method using a Laser Light Scattering Instrument (Mastersizer S, Malvern, UK). The dried microsphere samples were suspended in alcohol and sonicated before measurement. The obtained homogeneous suspensions were used to determine the mean diameter and diameter range. Each measurement was performed in triplicate.

Physical-chemical characterization

Physical-chemical properties of the microspheres and their components were analyzed by Fourier-transform infrared spectrometry (FT-IR) using a Perkin Elmer system 2000 spectrometer (USA). Samples were scanned from 400 to 4,000 cm^{-1} at a resolution of 4 cm^{-1}.

Results and discussion

Preparation and characterization of CHS–HA microspheres

Emulsification-coacervation is a facile method without complicated processing steps. In our study, CHS was

positively charged in acidic solution, while TPP and HA were negatively charged in water solution. Thus, double-walled CHS–HA microspheres were formed by the ionic interaction between positively charged amino groups of chitosan and negatively charged counterions of TPP and HA.

As we known, $-P_3O_{10}^{5-}$, $-HP_3O_{10}^{4-}$ and $-H_2P_3O_{10}^{3-}$ could coexist in the tripolyphosphate solution under all pH values [19]. However, the concentration of $-P_3O_{10}^{5-}$ was the highest, thus it was used to represent tripolyphosphate ions in current study for the convenience of description.

Sketch maps of CHS–HA microspheres fabrication process were shown in Fig. 1. When inner water phase met with outer water phase, anions $-P_3O_{10}^{5-}$ and $-COO^-$ reacted with cation $-NH_3^+$. In the beginning of this process, coacervation layer was formed at the surface of the CHS droplets. Then $-P_3O_{10}^{5-}$ could penetrate the coacervation layer and reached the core, and crosslinked with it. However, because HA has large molecular size and cannot penetrate the coacervation layer, it only reacted with $-NH_3^+$ at the surface. Double-walled microspheres were thus formed. Under different preparative conditions, microspheres with various shell thickness could be formed.

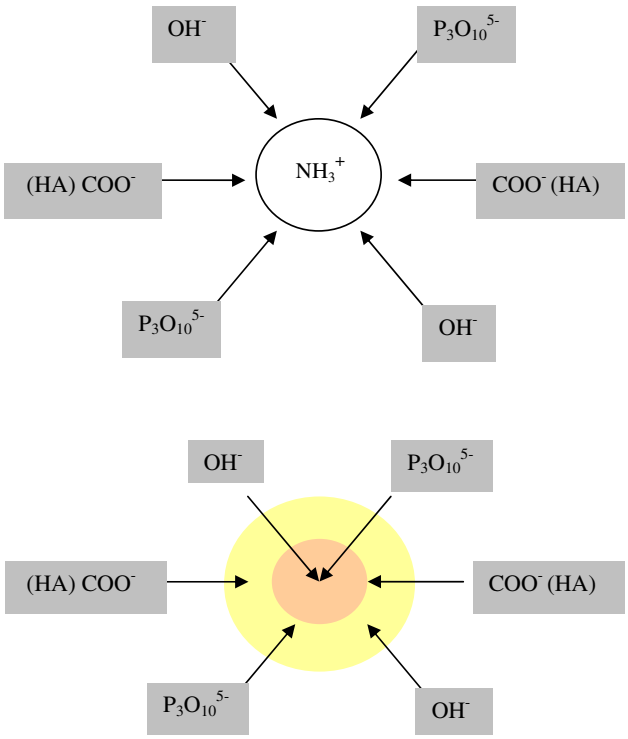
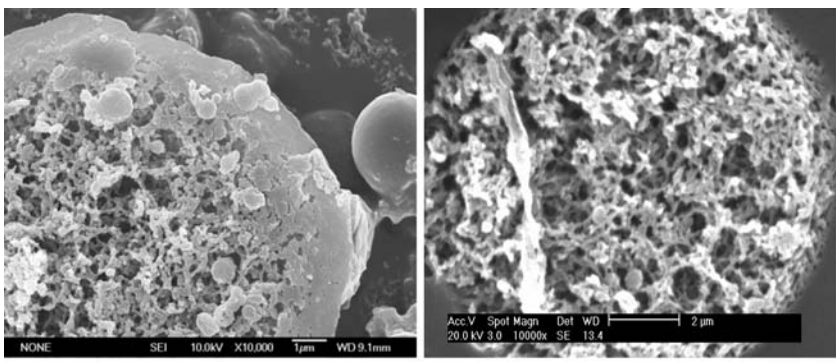


Fig. 1 Sketch maps of CHS–HA microspheres fabrication process

The morphology of microspheres was shown in Fig. 2. CHS–HA microspheres were spherical in shape and have smooth surfaces. The SEM of transversal section showed a finely porous core coated with an imperforate and compact layer, which indicated that double-walled microspheres were fabricated. The zeta potentials of CHS microspheres, CHS–TPP microspheres and CHS–HA microspheres supported the same conclusion (Table 1).

As shown in Fig. 3, the size of both CHS–TPP microspheres and CHS–HA microspheres approximately followed a Gaussian distribution, and the diameters were smaller than 40 μm. The mean size of CHS–HA microspheres and CHS–TPP microspheres was 12.19 μm and 10.51 μm respectively, with CHS–HA microspheres had a nearly 2 μm larger mean size compared with that of CHS–TPP microspheres.

To investigate the interactions between CHS, TPP and HA in the microphere formation process, FT-IR study was conducted. The FT-IR spectra of CHS, HA matrix and CHS–HA microspheres were shown in Fig. 4. A band at 3,450 cm^{-1} has been previously attributed to $-NH_2$ and –OH group stretching vibration in chitosan matrix [20]. The characteristic bands at 1,614 cm^{-1} and 1,405 cm^{-1} have been attributed to C=O group stretching vibration in HA matrix. For CHS–HA microspheres, there was a shift from 3,450 cm^{-1} to 3,412 cm^{-1} and the peak of 3,412 cm^{-1} became wider, which indicated that the hydrogen bonding was enhanced [21]. The shoulder peak of 1,649 cm^{-1} disappeared while a new sharp peak 1,630 cm^{-1} emerged and the 1,585 cm^{-1} peak of $-NH_2$ bending vibration shifted to 1,540 cm^{-1}. Because of $-NH_2$ bending vibration shifts, the characteristic bands at



CHS-HA microsphere CHS-TPP microsphere

Fig. 2 SEM photographs of intersectional CHS–HA microspheres and CHS–TPP microspheres

Table 1 Zeta potentials of the microspheres

Microsphere	Zeta potential (mV)
CHS	+13.6 ± 1.53
CHS–TPP	+5.3 ± 0.45
CHS–HA	
Oil/water volume ratio	
1:0.2	+4.5 ± 0.39
1:0.4	+2.4 ± 0.13
1:0.6	−4.0 ± 0.26
1:0.8	−3.6 ± 0.41
1:1.0	−4.0 ± 0.37
1:1.2	−5.9 ± 0.62
Solution pH	
4.0	+5.0 ± 0.27
5.0	+3.0 ± 0.23
6.0	−1.7 ± 0.18
7.0	−3.2 ± 0.40
8.0	−0.4 ± 0.06

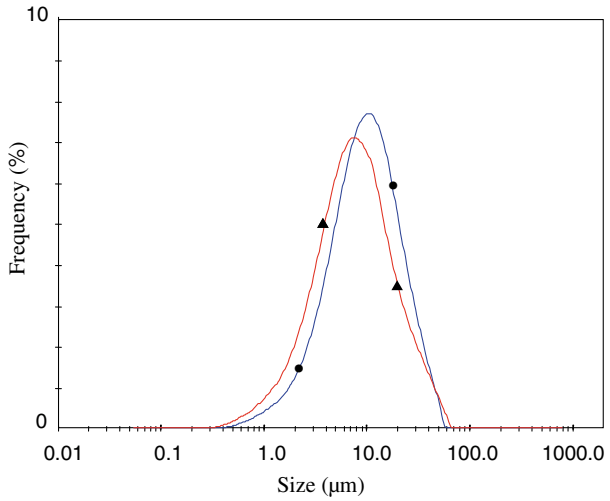


Fig. 3 Size distribution of CHS–TPP microspheres (▲) and CHS–HA microspheres (●)

1,614 cm^{-1} and 1,405 cm^{-1} shifted to 1,630 cm^{-1} and 1,391 cm^{-1} respectively. The presentation of P=O vibration absorption at 1,212 cm^{-1} was observed, which indicated the reaction between CHS and TPP. Similar results were reported by Knaul et al. in their study of chitosan film treated with phosphate (NaH_2PO_4) and they attributed this to the linkage between phosphoric and ammonium ion [22]. According to our results, we hypothesized that the $-P_3O_{10}^{5-}$ groups of TPP and $-COO^-$ of HA were linked with $-NH_3^+$ group of chitosan, and the inter and intramolecular actions were enhanced in CHS–HA microspheres.

The effect of oil/water volume ratio on microspheres formation

It has been reported that in emulsification-coacervation method, microspheres could be formed with different oil/water volume ratios [23, 24]. However, details of the influence of oil/water ratio on microsphere formation have not been systematically studied. In our study, 6 oil/water volume ratios, namely 1:0.2, 1:0.4, 1:0.6, 1:0.8, 1:1.0, and 1:1.2, was used to examine its effect.

SEM photographs of microspheres formed with different volume ratio were presented in Fig. 5. The microspheres formed on 1:0.2, 1:0.4 and 1:0.6 level were spherical, smooth, uniform and less aggregated. In contrast, when oil/water volume ratio further decreased, microspheres became irregular, coarse and more aggregated. Possible explanation for the observed differences was: when oil/water volume ratio was high, water droplets were totally surrounded by oil phase, and the oil/water emulsification was perfect, which could make water droplets separate from each other and keep spherical shapes. However, when oil/water volume ratio was low, water droplets were partly surrounded by the outer water phase, thus the droplets became aggregated and could not keep spherical shapes. From the above results, we could conclude that proper oil/water volume ratio was important to the morphology of microspheres.

Zeta potential is representative of particle charge. Compared with that of CHS–TPP microspheres (+5.3 mV), the zeta potentials of CHS–HA microspheres were much lower and were negative in most cases (Table 1). This was because that $-NH_3^+$ in the CHS droplets reacted with $-P_3O_{10}^{5-}$ and $-COO^-$, thus the surface zeta potentials decreased. As shown in Table 1, with decreasing oil/water volume ratio, the zeta potentials of CHS–HA microspheres decreased accordingly. This indicated that when the oil/water volume ratio was very high, there was less reaction between $-NH_3^+$ of the CHS droplets and $-P_3O_{10}^{5-}$, $-COO^-$ in outer water phase. As the volume of outer water phase increased, $-NH_3^+$ could meet more $-P_3O_{10}^{5-}$, $-COO^-$, so zeta potential of microspheres fell off.

The effect of surfactant on microspheres formation

Surfactant plays crucial role in microsphere preparation. It affected not only microsphere morphology, but also drug encapsulation efficiency and delivery properties [25–28]. Improved stabilization afforded by blend of surfactants versus single surfactants was commonly encountered and employed in the calculation of the required HLB (hydrophilic-lipophilic balance) of a system [29]. However, in our study, no advantage was observed for two-surfactant blend

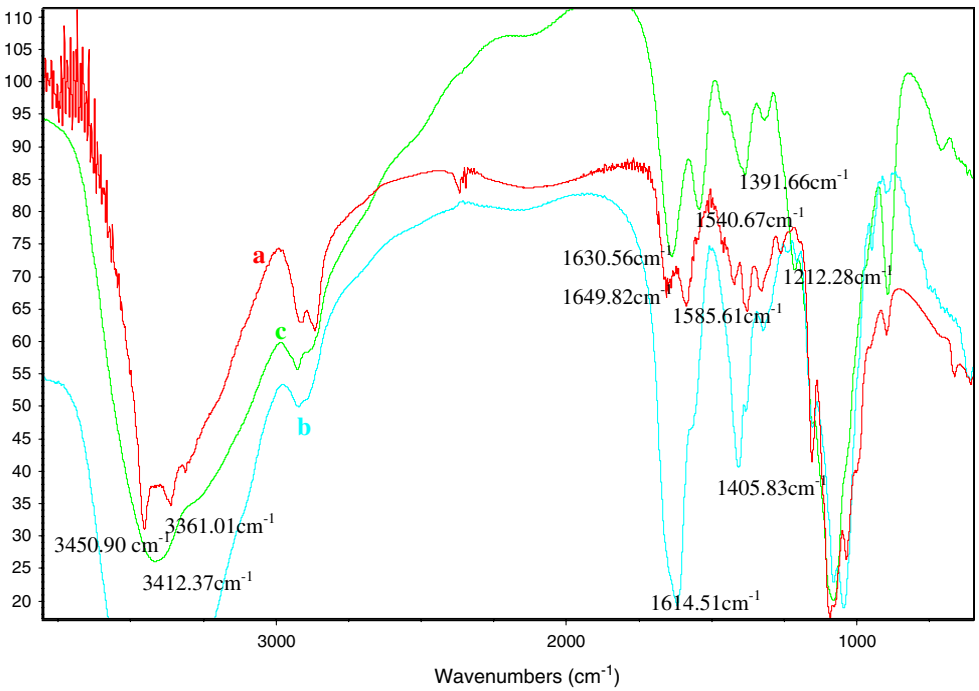


Fig. 4 FT-IR spectra of CHS matrix, HA matrix and CHS–HA microspheres: (**a**) CHS; (**b**) HA; (**c**) CHS–HA microspheres

compared with single surfactant. It was shown that microspheres made with only span80 in preparation process were spherical and less aggregated. In contrast, the microspheres made with both span80 and tween80 (at various concentrations) were irregular and more aggregated, and many gels were formed (Fig. 6). Similar phenomenon has been reported by Lim [30], but the author didn't provide any explanation.

It is interesting to discuss why two-surfactant blend had no advantage compared with single surfactant Span80. Firstly, although it is well accepted that mixture of surfactants may favor microspheres formation due to HLB change, there was study reported that HLB value had no relationship with surface morphology of microsphere [31]. Secondly, it was reported that surfactant could hinder the coacervation process among polymer [32]. So, in our study, when two surfactants were applied together, ion interaction between cations and anions may be hindered. Further works are needed to examine the underlying mechanism.

The effect of solution pH on microspheres formation

To investigate the effect of solution pH on microspheres formation, the pH of outer water phase was set at 4.0, 5.0, 6.0, 7.0 and 8.0 respectively with CHS solution pH fixed at a constant value 3.5.

When solution pH was higher than 7.0, only a small proportion of CHS amine groups were ionized [33]. A lot of $–OH^-$ and $–COO^-$ anions coexisted in the solution [19] and so a thick coacervation layer was formed, this thick layer could hinder $–P_3O_{10}^{5-}$ from diffusing into the CHS droplets and solidifying them. Also, because OH^- has small molecular size, it could diffuse into the core of CHS droplets and compete with $–P_3O_{10}^{5-}$ to react with limited $–NH_3^+$. Thus, the inner-crosslinkage of micropheres was insufficient, and CHS–HA microspheres with irregular shape were formed. In contrast, when pH was less than 7.0, $–OH^-$ concentration was very low and the concentration of $–COO^-$ was medium-high. At the same time, with decreasing pH value, more CHS amine groups were ionized. So there were more reactions between cation and anions, and compact and spherical double-walled microspheres were formed (Fig. 7). However, under stronger acidic conditions (e.g. pH < 4.0), although the concentration of $–NH_3^+$ was higher, because the anion amount ($–P_3O_{10}^{5-}$ and $–COO^-$) was too low, there was less interaction between cation and anions. FT-IR spectra showed that the intensity of P=O peak at 1,212 cm^{-1} increased with decreasing pH (Fig. 8). This was the same with the result reported from an earlier study [19] and could be attributed to the increase of interchain linkage of $–NH_3^+$ groups in chitosan by $–P_3O_{10}^{5-}$. As shown in Table 1, the surface charge increased with decreasing pH value, this was consistent with the changes of ions concentration.

In summary, solution pH was very important for microspheres fabrication. It controlled the charge numbers of cation and anion, and significantly influenced the extent of ionic-crosslinking density of CHS–HA microspheres.

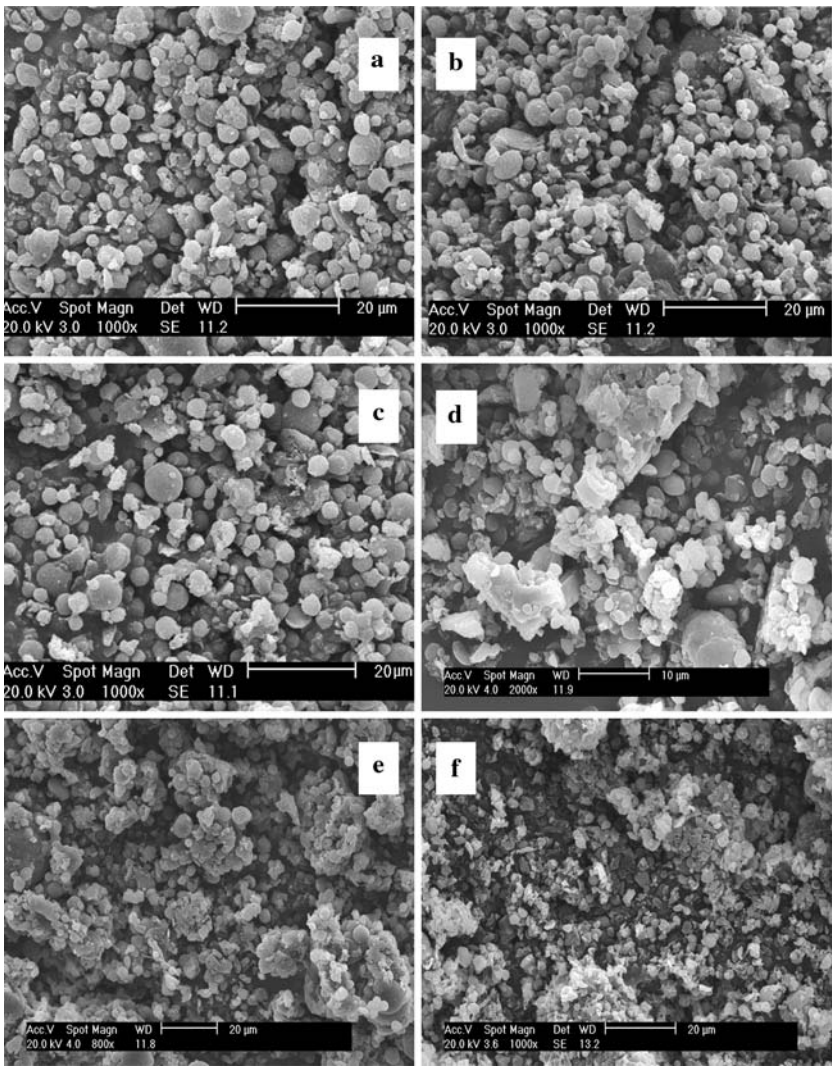


Fig. 5 SEM photographs of CHS–HA microspheres prepared with various oil/water volume ratios: (**a**) 1:0.2; (**b**) 1:0.4; (**c**) 1:0.6; (**d**) 1:0.8; (**e**) 1:1.0; (**f**) 1:1.2

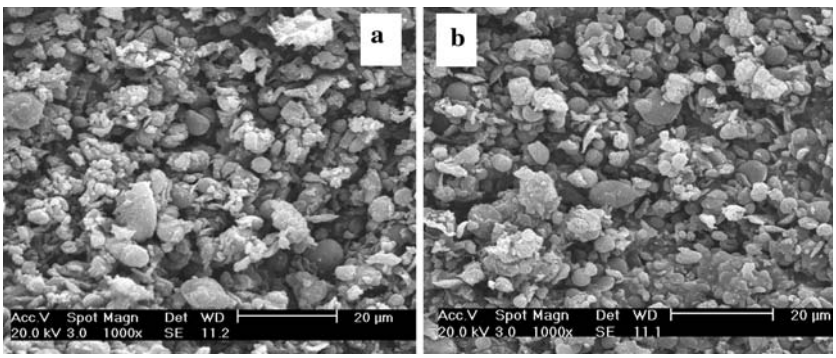


Fig. 6 SEM photographs of CHS–HA microspheres prepared with span80 and tween80 applied together: (**a**) when oil/water volume ratio was 1:0.2; (**b**) when oil/water volume ratio was 1:0.6

The effects of the concentration of TPP and HA on microspheres formation

TPP was the ion crosslinker, it could diffuse into the core of CHS droplets and crosslink with $-NH_3^+$. Since CHS core was the decisive part of CHS–HA microsphere, the concentration of $-P_3O_{10}^{5-}$ was crucial to microsphere preparation and morphology. In our study, various concentrations of TPP (1%, 3%, 5%, 8%, and 10%) were used. When TPP concentration was low, CHS droplets could not get sufficient crosslinkage, only few microspheres were formed and lots of gelatins existed. In addition, there was no obvious improvement when HA concentration changed because $-COO^-$ could not crosslink the CHS droplets core.

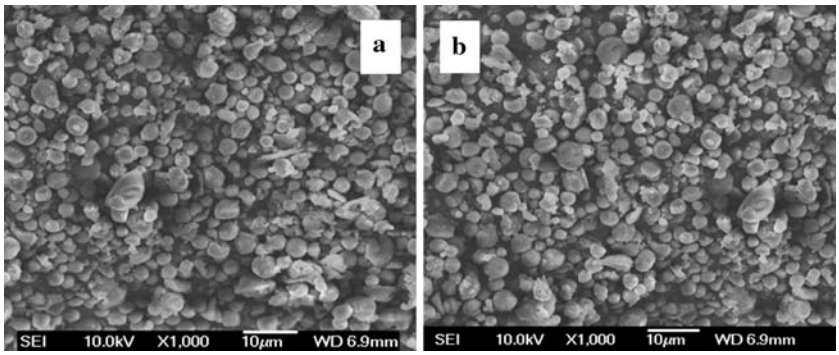


Fig. 7 SEM photographs of CHS–HA microspheres prepared at pH 6.0 and 7.0: (**a**) pH 6.0; (**b**) pH 7.0

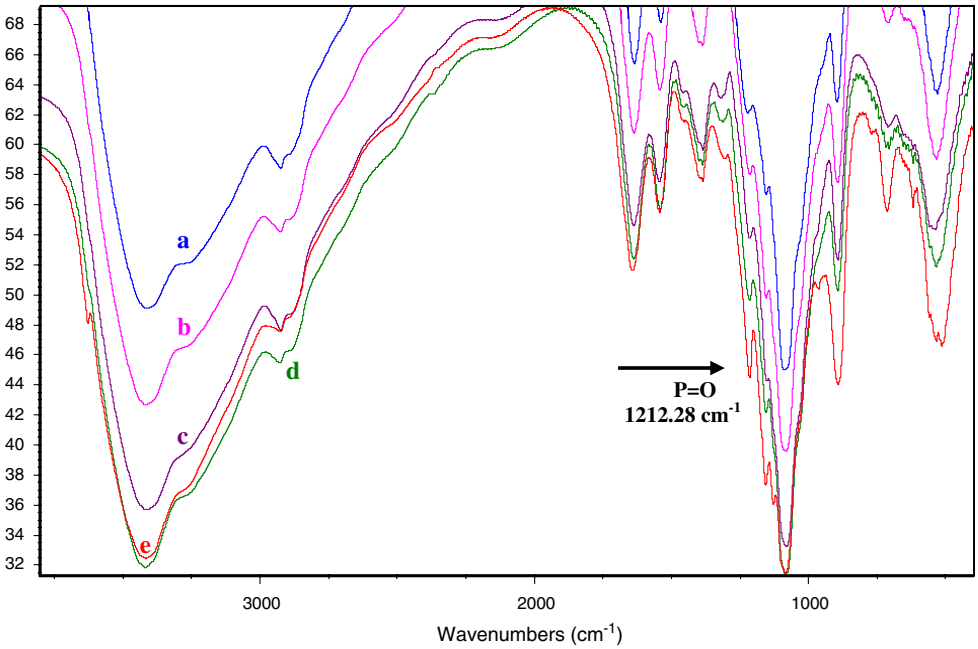


Fig. 8 FT-IR spectra of CHS–HA microspheres formed at different solution pH: (**a**) 8.0; (**b**) 7.0; (**c**) 6.0; (**d**)5.0; (**e**) 4.0

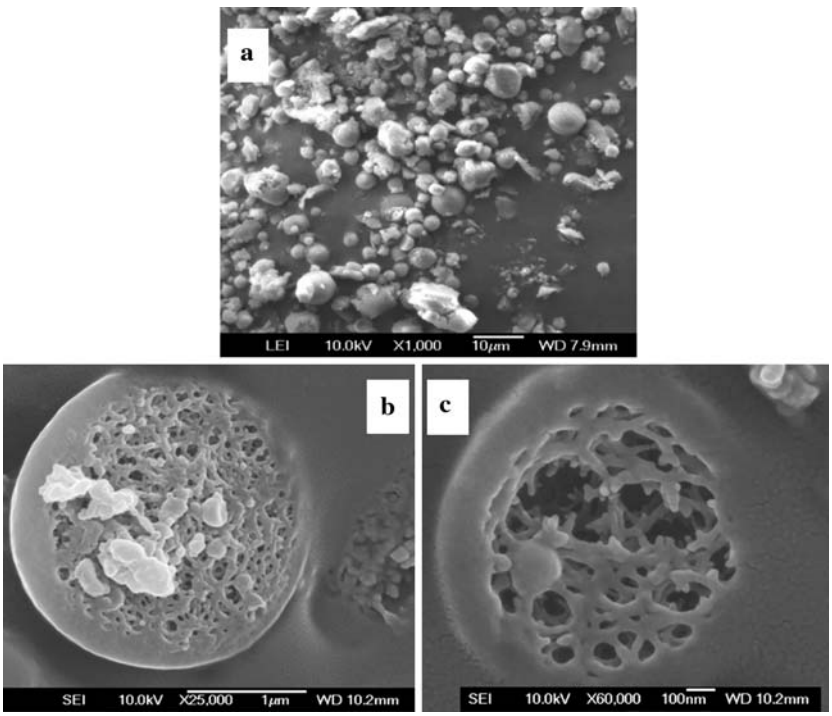


Fig. 9 Surface and intersection morphology of CHS–HA microspheres prepared at different HA concentrations: (**a**) surface morphology of microspheres when HA concentration was 0.4%; (**b**) intersection morphology of microspheres when HA concentration was 0.2%; (**c**) intersection morphology of microspheres when HA concentration was 0.4%

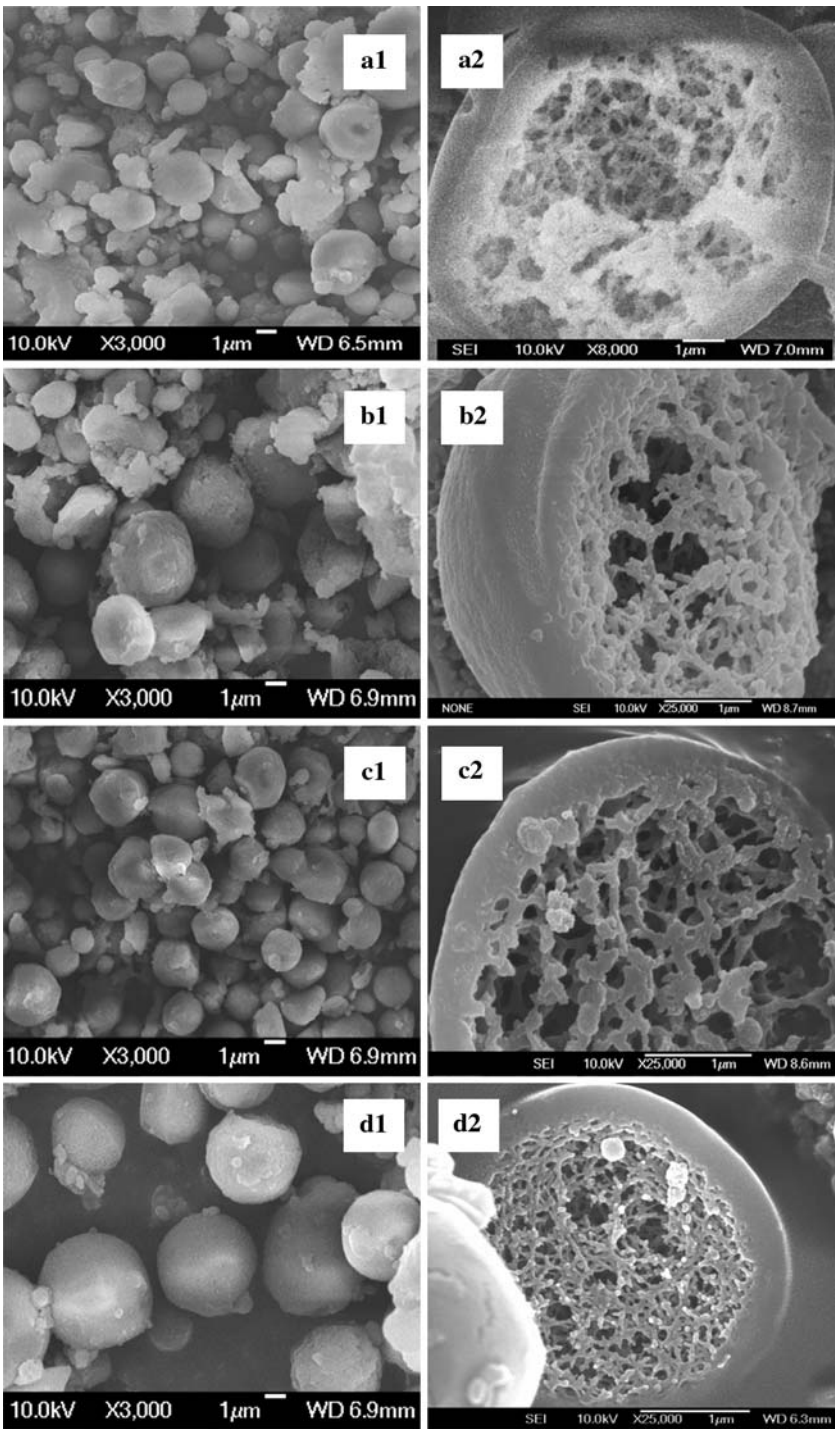


Fig. 10 Surface and intersection morphology of CHS–HA microspheres prepared at different emulsification time: (**a**) 0.5 h; (**b**) 1 h; (**c**) 2 h; (**d**) 3 h. a1, b1, c1 and d1 are surface morphology, a2, b2, c2 and d2 are intersection morphology

When TPP concentration increased to 8%, a lot of microspheres with spherical shape formed, and microspheres with the best morphology were obtained at a TPP concentration of 10%.

As for HA concentration, because $-COO^-$ only formed the outer shell of CHS–HA microspheres, it had less influence on CHS–HA microspheres morphology. When TPP concentration was 10%, little difference was observed for microspheres morphology when HA concentration changed in the 0.025% to 0.2% range. The zeta potentials of microspheres also had no obvious difference (results not shown) in this concentration range. However, when HA concentration increased to 0.4%, the morphology of microspheres became irregular and non-uniform, and an approximate microcapsule configuration rather than an impacted microsphere was formed (Fig. 9). This was

perhaps because that the viscosity of mixture solution was too high and thus the mixture couldn't reach full emulsification, and too much $-COO^-$ hindered $-P_3O_{10}^{5-}$ from penetrating the polyelectrolyte layer. FT-IR spectra also showed that for microspheres formed at HA concentration of 0.4%, the intensity of P=O peak at 1,212 cm^{-1} reduced compared with those formed at lower concentrations (figure not shown).

In summary, our results indicated that TPP concentration played a critical role on the preparation and morphology of CHS–HA microspheres, while HA concentration was relatively unimportant.

The effect of the emulsification time on microspheres formation

Emulsification time was another important factor affecting the formation of microspheres. As shown in Fig. 10, at 0.5 h stirring time, few microspheres were formed, and there were a great deal of gelatins (Fig. 10a). The microspheres were vacuous, irregular and had rough surfaces. With prolonging stirring time, microspheres became more spherical, smooth, and compact; the amount of gelatins also reduced. When stirring time increased to 3 h, the inter configuration and outer morphology of microspheres reach the best (Fig. 10d).

When HA concentration was low and solution pH was in $4 \sim 7$ range, microspheres with spherical shape were formed with short emulsification time. This is because that $-P_3O_{10}^{5-}$ could penetrate the polyelectrolyte layer easily, which ensured enough crosslinkage of CHS droplets. On the other hand, a lot of $-OH^-$ were generated when solution pH > 7.0, and $-COO^-$ concentration increased with increasing HA concentration. Those ions concentration changes made polyelectrolyte layer thicker, and then $-P_3O_{10}^{5-}$ needed longer time to reach the droplets core and solidified it. Therefore, with short emulsification time, only unshaped microspheres were formed.

So, sufficient emulsification time was necessary to get the spherical, smooth and less aggregated CHS–HA microspheres.

Bioactivity of protein drugs and CHS–HA double-walled microspheres

Because CHS–HA microspheres were prepared for protein drugs delivery, the bioactivity of protein drugs loaded should be kept well in the preparation process.

Protein is very sensitive to its surroundings and denaturation might be caused by oil/water interface and low-pH acid solutions [34]. In the beginning of CHS–HA microsphere preparation process, protein bioactivity could be lightly damaged by the oil/water interface when it was stirred, but this happened only in a very short time (15 mins), and addition of surfactants (span80) provided protection. After outer water phase was added, protein drug was encapsulated in the CHS core and covered by HA shell layer, it didn't confront the oil/water interface and further harm was avoided. Therefore, oil/water interface had little influence on protein bioactivity in this method and this is one advantage of double-walled microsphere. In our study, pH value of the inner water phase (protein drug existed in this phase) was not too low (pH 3.5), in addition, with the diffusion of the outer water phase into the inner droplet, the pH value of inner water phase increased, thus the protein bioactivity could not be affected much. In addition, several other factors such as ionic crosslinkage, low stirring rate, and lack of polar solvent also could contribute to the preservation of protein drug bioactivity in the preparation of CHS–HA microspheres by the emulsification- coacervation method.

Conclusions

CHS–HA double-walled microspheres were prepared by emulsification-coacervation method. TPP concentration, solution pH, surfactant, and emulsification time were important factors for micrpspheres formation. Emulsification-coacervation was a mild method and CHS–HA microspheres could have potential application as a delivery system for protein drugs.

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