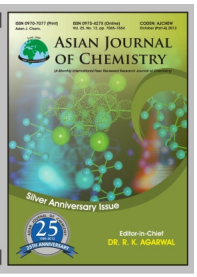
Asian Journal of Chemistry; Vol. 25, No. 18 (2013), 10368-10372

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15507



Preparation and Characterization of Chitosan-Harpin$_{Xooc}$ Nanoparticles

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(*Received*: 19 March 2013; *Accepted*: 14 November 2013) AJC-14405

The chitosan-Harpin$_{Xooc}$ nanoparticles were prepared by the electrostatic interaction of chitosan (CS) with Harpin$_{Xooc}$ and the ionic gelation of chitosan with tripolyphosphate (TPP). The particle sizes (197-653 nm) and zeta potential (29-15 mV) of the nanoparticles, which were changed with the preparing conditions such as the pH value (4.8-6.8), CS/TPP mass ratio (20:1-6:1) and Harpin$_{Xooc}$ concentration (50-400 µg/mL), were determined respectively by dynamic light scattering and zeta potential analyzer. These preparing conditions were also examined systematically for their effects on the particles physicochemical properties such as encapsulation efficiency, loading capacity and release behavior at 26 ± 2 °C. The morphology and microstructure of the chitosan-tripolyphosphate nanoparticles were revealed by the transmission electron microscope analysis. This study demonstrated that systematic design and modulation of the chitosan-Harpin$_{Xooc}$ nanoparticles could be readily achieved with the right control of processing conditions and would provide a sound basis to further exploration of Harpin$_{Xooc}$ loaded nanoparticles.

Key Words: Harpin$_{Xooc}$, Chitosan, Nanoparticles.

INTRODUCTION

Harpin$_{Xooc}$ from rice pathogenic bacterium *Xanthomonas oryzae* pv. *oryzicola* has been investigated for biocontrol due to its ability of eliciting hypersensitive cell death in non-host plants, inducing disease and insect resistance in plants and enhancing plant growth[1-3]. The Harpin$_{Xooc}$ has been developed as the commercial agent Yilite[4] and has good effect on rice and tobacco[3-5].

In recent years, nanoparticles were applied in various areas including biomedicine and pharmaceuticals as a formulation[6,7], owing to their high volume/surface ratio, surface tailorability, improved solubility and multi-functionality[8]. Among various carriers, chitosan sodium tripolyphosphate (TPP) nanoparticles as drug sustained-release carrier is getting more and more attention for protein molecules[9]. Because the chitosan-TPP system forms under mild conditions, is homogeneous with adjustable size and possess positive surface charge that can be easily modulated by varying the processing conditions[9]. It is necessary and promising to explore the characteristic of Harpin$_{Xooc}$ loaded nanoparticles for intended applications.

In this study, the Harpin$_{Xooc}$ nanoparticles based on chitosan was prepared by the ionic gelation and characterized.

EXPERIMENTAL

For obtaining obtained purified Harpin$_{Xooc}$ easily, 6-histidine-tagged Harpin$_{Xooc}$ protein was used. Harpin$_{Xooc}$ protein was extracted from BL21[pET30(a+)::hrf2] (constructed in previous study).We used HisTrap Kit (GE Healthcare Biosciences Ltd.) to obtain highly purified Harpin$_{Xooc}$[1]. Purified protein was dialyzed for 36 h and detected by SDS-PAGE. The isoelectric value (pI) of Harpin$_{Xooc}$ was about 4.7. Protein concentration was measured with Bradford reagent using BSA as the standard protein molecular.

Chitosan (CS, poly-[(1-4)-linked-2-amino-2-deoxy-D-glucose], MMW, the degree of deacetylation was 75 %-85 %) was purchased from Aldrich and the Brookfield viscosity was 200 cps. Sodium tripolyphosphate (TPP, AR) was purchased from Sangon Co. Ltd. (Shanghai, China). All other reagents were reagent grade.

Conditions for the formation of chitosan nanoparticles: Chitosan-sodium tripolyphosphate nanoparticles (CS-TPP) were prepared according to the procedure of Calvo *et al.*[10] based on the ionic gelation of chitosan with sodium tripolyphosphate (TPP) anions. Preliminary experiments were done in order to determine the production zone of the nanoparticles formation. chitosan was dissolved in 1 % acetic acid solution, stirred for 2 h and filtered by paper filter. 0.3 mL TPP (dissolved in

purified water) at certain concentration was dropped into 1 g/mL chitosan solution with a syringe under stirring. By adjusting pH or TPP amount, three different systems were obtained: clear solution, opalescent suspension and aggregates. The opalescent suspension should correspond to a suspension of nanoparticles. This opalescent system was used for further experiment of Harpin$_{Xooc}$ protein loading and release.

Preparation of CS-H-TPP nanoparticles: Protein loaded chitosan nanoparticles system can be done by two methods: incorporation or incubation[11]. In this study, we choose incorporation method for preparing nanoparticles with smaller size and larger zeta potential. Firstly, 0.3 mL Harpin$_{Xooc}$ was premixed with 2.4 mL 1 g/mL chitosan solution. The formation of Harpin$_{Xooc}$ loaded nanopartocles (CS-H-TPP) started when 0.3 mL TPP solution at some concentration was dropped into CS-Harpin$_{Xooc}$ solution with adjusting pH and stirring for 1 h. During the incorporation process, protein molecules were entrapped/embedded in the CS-H-TPP nano-matrix, with some protein molecules also absorbed at the particles surface. All nanoparticles suspension were prepared at 26 ± 2 °C.

In this work, CS-H-TPP was prepared based on electrostatic interaction. Proteins with a low electrostatic value are generally better encapsulated at a pH above pI value. Protein encapsulation in this work was all conducted at pH above 4.7.

Physicochemical characterization of nanoparticles: The size and zeta potential of the nanoparticles were measured at 25 °C with Zetasizer Nano ZS (Malvern instruments, UK). Triplicate samples were analyzed.

Morphological characteristics of the nanoparticles were examined using a transmission electron microscope (TEM) machine (H-7650, HITACH, Japan). Freshly made Nanoparticles were diluted with deionized water. One-drop sample was placed on a carbon coated film copper grid and stained with phosphotungstic acid (PTA) for 1 min. They were dried and examined by TEM.

Determination of encapsulation efficiency and loading capacity: To determine the encapsulation efficiency (EE) and loading capacity (LC), Harpin$_{Xooc}$ nanoparticles were separated from the aqueous suspension medium by ultra centrifugation with 20000 rpm at 25 °C for 0.5 h. The sediments were freeze-dried over 48 h and weighted up. The amount of free Harpin$_{Xooc}$ in the supernatant was measured with Bradford reagent, using the supernatant of the CS-TPP nanoparticles as basic correction. Each sample was repeatedly measured 4 times. The encapsulation efficiency and loading capacity of the nanoparticles were calculated with the following equation[12]:

$$EE\ (\%) = (\text{total Harpin}_{Xooc}\text{-free Harpin}_{Xooc})/\text{total Harpin}_{Xooc} \times 100$$

$$LC\ (\%) = (\text{total Harpin}_{Xooc}\text{-free Harpin}_{Xooc})/\text{nanoparticles weight} \times 100$$

Harpin$_{Xooc}$ releasing from the nanoparticles *in vitro*: The protein release profiles of nanoparticles were determined as follows[11]: The nanoparticles in the form of sediments were incubated in 3 mL phosphate buffered saline (PBS, pH 6.3) at 26 ± 2 °C under gently stirring. At predetermined time intervals, the samples were ultra-centrifuged, 100 µL supernatant was taken from the tube and the protein concentration was measured. The samples in the tube were replenished with 100 µL fresh PBS solution. Each sample was repeatedly measured 4 times. The total released protein mass M_i at time i was calculated from the following equation:

$$M_i = C_i V + \Sigma C_{i-1} V_s$$

where C_i is the concentration of protein in the release solution at time i, V the total volume of release solution and V_s is the sample volume.

RESULTS AND DISCUSSION

In an acidic medium, the amine groups of chitosan will be positively charged and the surface change density of chitosan molecules is strongly dependent on solution pH value[13,14] and the ionic cross-linking process for the formation of CS-TPP nanoparticles is pH-responsive, providing opportunities to modulate the formulation and properties of the CS-TPP nanoparticles. The effects of solution pH on CS-TPP nanoparticles size and zeta potential were studied by Gan *et al.*[9] This study characterized the CS-H-TPP nanoparticles for further use on agriculture.

The variations on particle size and zeta potential with solution pH are shown in Table-1. The average particle size of the CS-TPP or CS-H-TPP is increased with solution pH. The increase in average particle size could be caused mainly by particle aggregation when solution pH value increased, rather than by further growth of the individual particle size after initial formation[9]. The sharp increase in size of CS-H-TPP nanoparticles at pH > 6.3 suggests that the degree of protonisation at surface of particles were reduced, decreasing electrostatic repulsion between the particles thereby increasing the probability of particle aggregation. The deprotonisation of the particle surface was supported by zeta potential which decreased prominently at pH > 6.3. Fig. 1 shows the effect of

TABLE-1
PHYSICOCHEMICAL PROPERTIES OF HARPIN$_{Xooc}$ NANOPARTICLES PREPARED AT DIFFERENT pH

pH	CS-TPP	CS-H-TPP	
	Particle size (nm)	Particle size (nm)	Zeta potential (mV)
4.8	151.5 ± 2.5	196.9 ± 0.6	28.93 ± 0.6
5.3	157.8 ± 0.2	232.1 ± 0.1	22.63 ± 0.5
5.8	222.8 ± 10.9	267.2 ± 2.2	21.80 ± 1.4
6.3	243.1 ± 2.0	270.0 ± 3.0	16.53 ± 0.9
6.8	N.D.	566.6 ± 33.1	9.38 ± 0.9

Particle preparation conditions: CS/TPP mass ratio = 10:1, Harpin$_{Xooc}$ concentration = 100 µg/mL, T = 25 ± 2 °C.

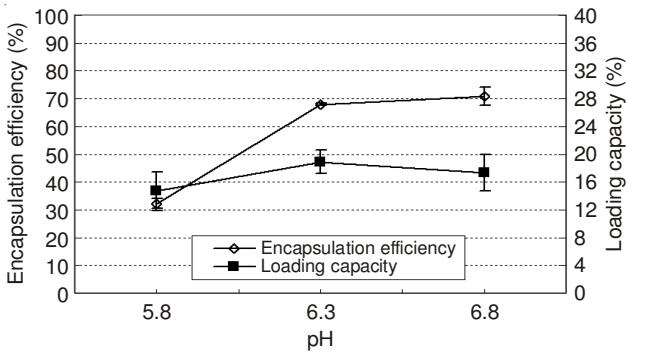

Fig. 1. Encapsulation efficiency and loading capacity of Harpin$_{Xooc}$ nanoparticles prepared at different pH. Particle preparation conditions: CS/TPP mass ratio=10:1, Harpin$_{Xooc}$ concentration = 200 µg/mL, T = 25 ± 2 °C

solution pH on encapsulation efficiency (EE) and loading capacity (LC) of nanoparticles. The encapsulation efficiency increased from 32.3 to 70.9 % when pH increased from 5.8 to 6.8, the loading capacity did not change abviously.

Effect of the mass ratio of CS to TPP: CS-TPP was prepared according to the procedure based on the ionic gelation of CS with TPP anions. In order to investigate the feasibility of preparing CS-H-TPP nanoparticles by ionic gelation, we prepared nanoparticles with CS/TPP mass ratio of 20:1 to 6:1. The solution was clear or aggregation formation when the CS/TPP ratios were higher than 20:1 or lower than 6:1 (data not shown).

Table-2 shows the influence of CS/TPP ratios on particle sizes and zeta potential values of nanoparticles (CS-TPP and CS-H-TPP). A slight increase in particle size and decrease in zeta potential with the decrease in CS/TPP ratio is noted. These relationships could be explored for manipulating and optimizing the nanoparticles size for intended applications and for modulating the particle surface charge density to facilitate the adhesion and transport properties of the nanoparticles[9].

TABLE-2
PHYSICO-CHEMICAL PROPERTIES OF HARPIN$_{Xooc}$ NANOPARTICLES PREPARED AT DIFFERENT CS/TPP MASS RATIO

CS/TPP Mass ratio	CS-TPP	CS-H-TPP	
	Particle size (nm)	Particle size (nm)	Zeta potential (mV)
20:1	171.0 ± 7.6	261.9 ± 5.2	18.67 ± 0.3
14:1	164.5 ± 0.3	265.7 ± 3.0	18.50 ± 0.5
10:1	243.1 ± 2.0	270.0 ± 3.0	16.53 ± 0.9
8:1	265.0 ± 7.4	290.5 ± 1.4	16.30 ± 0.4
6:1	301.2 ± 0.9	379.1 ± 2.6	14.90 ± 0.7

Particle preparation conditions: Harpin$_{Xooc}$ concentration = 100 μg/mL, pH 6.3, T = 25 ± 2 °C.

Reports on CS/TPP mass ratio effect on size are inconclusive and sometimes contradictory. Our result is in agreement with studies carried out by Calvo *et al.*[10] and Wang *et al.*[12] at pH 7.4 or near 4.0, but is in contrary to Gan *et al.*[9,11] and Grenha[15] who reported the reversed results at different pH. This difference may be relative to the preparing method. One method was that TPP was added to CS solution, the other TPP was mixed with CS solution.

The effects of CS to TPP mass ratio on encapsulation efficiency and loading capacity were studied at the mass ratios of 6, 8, 10, 12 and 20 at pH 6.3. Results presented in Fig. 2 showed encapsulation efficiency increased from 59.8 to 72.8 % and loading capacity decreased from 29.9 to 15.1 % when CS to TPP mass ratios increased.

Effect of Harpin$_{Xooc}$ concentration: Table-3 demonstrated that the particle size of nanoparticles increased gradually as the Harpin$_{Xooc}$ concentration increased from 50 to 400 μg/mL. As expected, the zeta potential decrease when Harpin$_{Xooc}$ concentration increase. Big aggregation formed when Harpin$_{Xooc}$ concentration was above 400 μg/mL. When Harpin$_{Xooc}$ concentration was 50 μg/mL, the nanoparticle had smaller particle size and larger zeta potential. These parameters were conducted for further use on agriculture which still should be optimized in further field such as eliciting hypersensitive cell death and

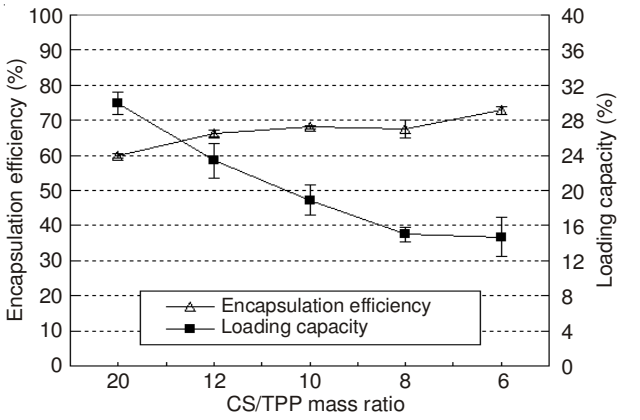

Fig. 2. Encapsulation efficiency and loading capacity of Harpin$_{Xooc}$ nanoparticles prepared at different CS/TPP mass ratio. Particle preparation conditions: pH 6.3, Harpin$_{Xooc}$ concentration = 200 μg/mL, T = 25 ± 2 °C

TABLE-3
PHYSICO-CHEMICAL PROPERTIES OF HARPIN$_{Xooc}$ NANOPARTICLES PREPARED AT DIFFERENT HARPIN$_{Xooc}$ CONCENTRATION

Harpin$_{Xooc}$ concentration (μg/mL)	Particle size (nm)	Zeta potential (mV)
50	231.4 ± 4.7	18.47 ± 0.6
100	270.0 ± 3.0	16.53 ± 0.9
200	285.2 ± 3.5	15.70 ± 0.7
300	320.7 ± 4.1	15.30 ± 0.9
400	653.4 ± 27.6	14.90 ± 1.3

Particle preparation conditions: pH6.3, CS/TPP mass ratio = 10:1, T = 25 ± 2 °C

enhancing plant growth. The effects of the Harpin$_{Xooc}$ concentration on encapsulation efficiency and loading capacity were studied at the concentration of 100, 200, 300, 400 and 500 μg/mL at pH 6.3. Results presented in Fig. 3 showed encapsulation efficiency increased from 54.2 to 89.5 % and loading capacity increased from 12.6 to 33.9 % when the Harpin$_{Xooc}$ concentration increased.

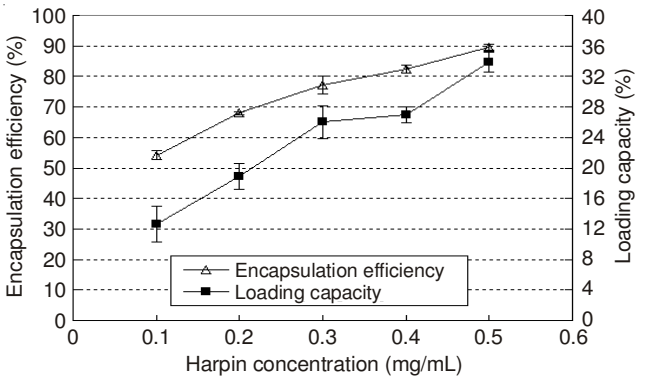

Fig. 3. Encapsulation efficiency and loading capacity of Harpin$_{Xooc}$ nanoparticles prepared at different Harpin$_{Xooc}$ concentration. Particle preparation conditions: pH = 6.3, CS/TPP mass ratio = 10:1, T = 25 ± 2 °C

Release of Harpin$_{Xooc}$ from nanoparticles: As shown in Figs. 4 and 5, both release kinetics and especially total release at isothermal conditions decrease with decreasing CS to TPP mass ratio and pH. When the solution pH increased from 5.8 to 6.8, which is due to the optimal degree of swelling and

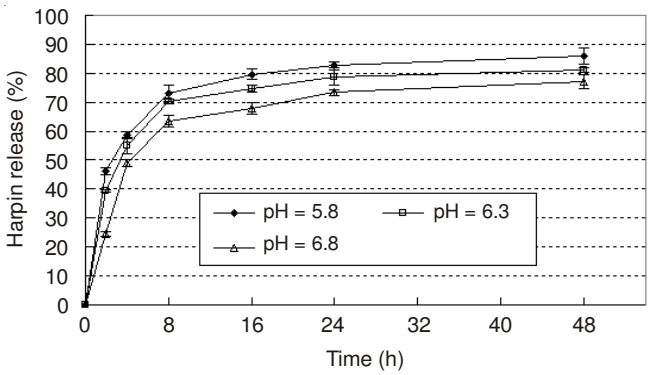

Fig.4. Harpin$_{Xooc}$ release profiles from harpin loaded nanoparticles at different pH. Harpin$_{Xooc}$ initial concentration = 200 μg mL^{-1}

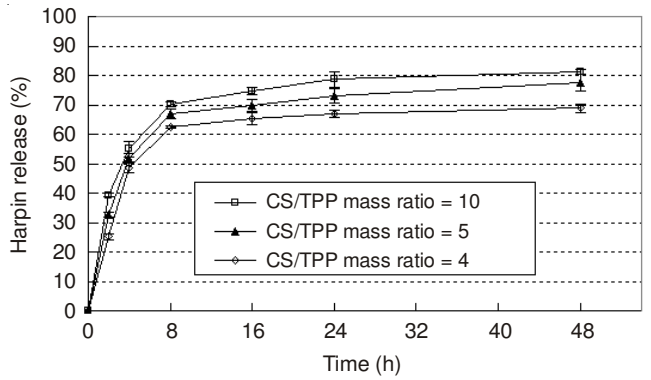

Fig. 5. Harpin$_{Xooc}$ release profiles from Harpin loaded nanoparticles at different CS/TPP mass ratio. Harpin$_{Xooc}$ initial concentration = 200 μg mL^{-1}

electrostatic interactions between TPP anions and chitosan[16]. Addition of TPP may provided a greater crosslink density with CS, causing increased chin packing and rigidity, as well as increased inter-chain bonding, thereby reducing harpin release from nanoparticles[11]. However, there always are amount of protein release burst at 8 h, because that protein departed from the nanoparticles' surface. Subsequently, Harpin$_{Xooc}$ released slowly from nanoparticles due to the optimal degree of swelling and electrostatic interactions between TPP anions and chitosan[16].

TEM observation of nanoparticles: From TEM image (Fig. 6a), well dispersed CS-TPP nanoparticles with small size were observed (pH = 3.3, CS/TPP mass ratio is 4). The structure became solid and big when the CS/TPP mass ratio was 2, because more TPP reacting ionically with chitosan resulted the solubility decreasing and the nanoparticles become large[17]. Fig. 6c showed a image of CS-TPP nanoparticles at CS/TPP mass ratio = 10. The particles become irregular and the size increase nearly 200 nm.

Conclusion

Harpin$_{Xooc}$ can elicit hypersensitive cell death in non-host plants, induce disease and insect resistance in plants and enhance plant growth. There is a window of preparation and systematically manipulating the conditions of Harpin$_{Xooc}$ loaded nanoparticles. The formation of polyelectrolyte chitosan-Harpin$_{Xooc}$-TPP nanoparticles with well nano metric size can be simply manipulated and controlled by varying the processing conditions, including chitosan to TPP weight ratio,

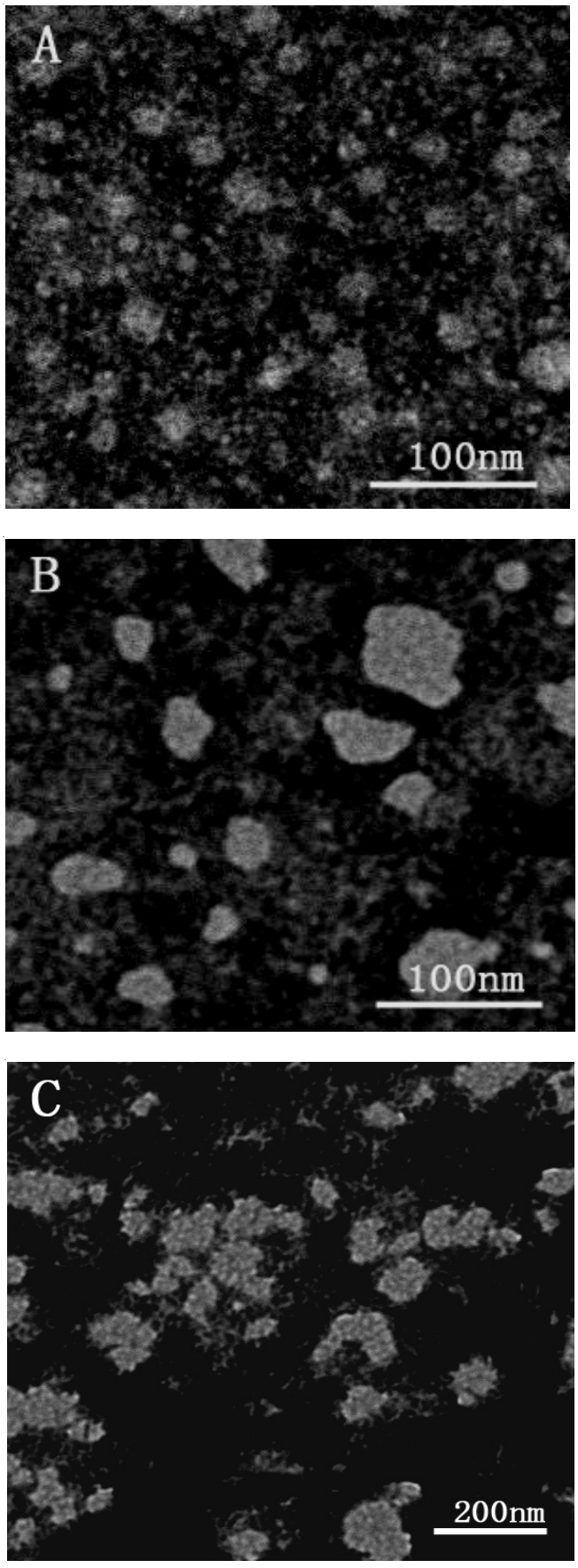

Fig. 6. TEM image of CS nanoparticles at different preparing conditions. (a) pH = 3.3, CS/TPP mass ratio = 4 (b) pH = 3.3, CS/TPP mass ratio = 2 (c) pH = 6.0, CS/TPP mass ratio = 10

solution pH and protein concentration. However, manipulation of these conditions did not succeed in controlling the typical burst release, although, the release profile of harpin from nanoparticles has a slowly continuous release phase followed. The sequential TEM images provided a useful morphological observation for chitosan-TPP nanoparticles. Simultaneity, systemic work should be proceeded to demonstrate its effects on plants. Furthermore, it is necessary to develop better nanoscale pesticide form to promote application on agriculture.

ACKNOWLEDGEMENTS

This work was supported by grants from the National High-tech R&D Program of China (2012AA101504), the Program of International Science and Technology Cooperation (2009DFA32740), the Special fund for Agro-scientific Research in the Public Interest (20130315) and the National Transgenic Major Program (2011ZX08004-004)

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