Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 62 (2005) 293–298
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Self-assembled nanoparticles based on linoleic-acid modified chitosan: Stability and adsorption of trypsin

Chen-Guang Liu[a,b], Xi-Guang Chen[a], Hyun-Jin Park[b,*]

[a]*The Life Science College, Ocean University of China, Qingdao 266003, People's Republic of China*
[b]*The Graduate School of Biotechnology, Korea University, 1, 5-Ka, Anam-Dong, Sungbuk-Ku, Seoul 136-701, South Korea*

Received 9 April 2005; revised 29 July 2005; accepted 2 August 2005
Available online 13 September 2005

Abstract

The hydrogel nanoparticles can be prepared using linoleic modified chitosan following sonication. The morphology and size distribution of nanoparticles were determined by AFM and photocorrelation spectroscopy, respectively. The effects of environment factors on particle size and adsorption of trypsin (TR) were also analyzed. The LA-chitosan nanoparticles were in the range of 200–600 nm with satisfactory structural integrity. The particle size slightly increased with an increase in concentration. In high ionic strength solution the particle size decreased, compared to salt-free solution, on the other hand, the particles size increases as urea concentration increased. The particle size was found to be larger in acidic solution than in neutral and alkaline solution. Environment factors (e.g. pH, concentration of urea or NaCl) can affect TR loading on the nanoparticle. The thermal stability of TR loading on nanoparticles was significantly improved compared to free TR.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Hydrophobically modified chitosan; Nanoparticles; Trypsin; Adsorption

1. Introduction

Amphiphilic polymers comprise a class of water-soluble polymers consisting of both hydrophobic and hydrophilic segments in a chain. According to Akiyoshi (2002) amphiphilic polymers can be classified into three groups: amphiphilic block copolymers, amphiphilic random copolymers and hydrophobically modified water-soluble polymers. When they are dissolved in water, these polymers can self-aggregate due to their intra- and/or intermolecular hydrophobic interaction. By this process, polymer micelles or nanoparticles with hydrophobic core and hydrophilic shell can be prepared, where hydrophobic segments are segregated from the aqueous exterior to form an inner core surrounded by a palisade of hydrophilic segments. This kind of structure is suitable for trapping hydrophobic substances, such as fluorescent probes and various proteins under mild conditions (Akiyoshi & Sunamoto, 1996). In particular, such amphiphilic polymer micelles or nanoparticles are unique hosts for protein or enzyme. Harada et al. (1998, 2001) and Jaturanpinyo et al. (2004) reported that a polyion micelle prepared from poly (ethylene glycol)-poly (aspartic acid) block copolymer can entrap the enzyme molecule (lysozyme, trypsin). They found an accelerated enzymatic reaction for this lysozyme/PEG-P (Asp) micelles system. Nishikawa et al. (1994) and Akiyoshi et al. (1996, 1998) have synthesized and characterized cholesteryl derivatives of pullulan, which undergo self-aggregation in water to form hydrogel nanoparticles. They showed the nanoparticles coupled stable complexation with insulin, BSA, and α-chymotrypsin.

Chitosan is a multi purpose material that has found a wide range of applications starting from dietary regime constituent, food packaging material, drug release component and for environmental pollutants among others (Arvanitoyannis et al., 1998; Arvanitoyannis, 1999; Sashiwa & Aiba, 2004; Sashiwa, Fujishima & Yamano, 2003). Recently, preparation of hydrogel nanoparticles based on hydrophobically modified chitosan has attracted research interest (Kwon, Park, Chung, Kwon, Jeong, & Kim, 2003; Lee, Jo, Kwon, Kim, & Jeong, 1998a, b) because chitosan has unique characteristics such as positive charge, nontoxicity, and bioadhesivity. Self-aggregated

* Corresponding author. Tel.: +82 2 3290 3450; fax: +82 2 9535 892.
E-mail address: hjpark@korea.ac.kr (H.-J. Park).

0144-8617/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2005.08.010

nanoparticles from hydrophobically modified chitosan (HMC) can be used as a DNA delivery system and anticancer drug (adriamycin) delivery carriers (Kim, Gihm, & Park, 2001; Lee, kim, Kwon, Kim, & Jeong, 2000; Liu, Zhang, Sun, Sun, & Yao, 2003). However, there are few reports on the use of HMC as peptide or protein carriers (Park, et al., 2004). In our previous work (2005), linolenic acid modified chitosan can form nanoparticles in pH 7.4 phosphate buffered saline (PBS) buffer after sonication, which can be used as protein (BSA) carrier. The mechanism of association between the protein and carrier need to be studied further. In this study, our object was to determine the morphology of nanoparticles based LA-CS and to investigate their stability in aqueous medium, interactions with enzyme (trypsin) and the activity of TR on nanopaticles.

2. Materials and methods

2.1. Materials

Chitosan (low molecular weight, Aldrich). Linoleic acids (LA), 1-ethyl-3-(3-dimethylaminopropyyl) carbodiimide (EDC), *N*(alpha)-benzoyl -DL-arginine-4-nitroanilide hydrochloride(BAPA), trypsin (TR) were purchased from Sigma Chemicals (St Louis, MO, USA).

2.2. Preparation of LA-chitosan

Linoleic acid was coupled to chitosan by the formation of amide linkages through the EDC-mediated reaction according to the method described by Chen, Lee, and Park, et al. (2003). Chitosan (1 g) was dissolved in 1% (w/v) aqueous acetic acid solution (100 mL) and diluted with 85 mL methanol. Linoleic acid was added to the chitosan solution at 0.54 mol/mol glucosamine residue of chitosan followed by a drop-wise addition of 15 mL EDC methanol solution (0.07 g/L) while stirring at room temperature. The 1:1 mol ratio of EDC to linoleic acid was used in this study. After 24 h, the reaction mixture was poured into 200 mL of methanol/ammonia solution (7/3, v/v) with stirring. The precipitated material was filtered, washed with distilled water, methanol, and ether and then dried under vacuum for 24 h at room temperature.

2.3. Particle size distribution

The average particle size and size distribution were determined by quasielastic laser light scattering with a Malvern Zetasizer® (Malvern Instruments Limited, UK) (Chung, Kim, Kwon, Kwon, & Jeong, 2001). Nanoparticle distilled water solution of 3 mL (1 mg/mL) were put into polystyrene latex cell and measured at a detector angle of 90°, wave length of 633 nm, refractive index of 1.33, real refractive index of 1.59 and temperature of 25 °C.

2.4. Atomic force microscopy (AFM)

The surface morphology of nanoparticles was observed using atomic force microscopy (AFM), for which a silicon nitride tip on a cantilever with a spring constant of 0.12 N/m was used. The nanoparticle solutions in distilled water (0.5 mg/mL) were used for taking measurements. The images were obtained under ambient conditions with the noncontact mode.

2.5. Determination of TR loading capacity

The TR solution was added to 2 mL nanoparticle solution in such a way that the concentration range of TR ranged between 100 and 2000 μg/mL. Thus, prepared solutions were incubated at 4 °C for 12 h. Then, these solutions were centrifuged at 30,000 *g* and 4 °C for 30 min to separate the unloaded TR (supernatant) and TR loaded nanoparticles. The unloaded TR present in supernatant was determined by UV spectroscopic method (280 nm) using supernatant solution of non-loaded nanoparticle solution as a blank. The TR loading capacity (LC) of nanoparticles was calculated by using following equation (Calvo, Remunan--Lopez, Vila-Jato, & Alonso, 1997):

$$\mathrm{LC} = (A - B)/C \times 100$$

A, total amount of BSA in added solution; *B*, total amount of BSA in supernatant after centrifugation; *C*, Weight of the nanoparticles measured after being freeze dried.

2.6. Enzyme activity determination

One millimolar solution of the PABA was prepared by dissolving substrate in 90 °C distilled water and making up the final volume with distilled water at 25 °C. The reaction mixture contained 50–60 μg of TR or 200 μg nanoparticle-TR complex and 2 mL of PABA. After 2 min of incubation at 28 °C, the reaction was terminated by an addition of 0.4 mL of 60% acetic acid. The liberated *p*-nitroaniline was monitored spectrophotometrically by measuring absorbance at 410 nm against a blank with no enzyme. An extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroaniline was used for calculations of specific activity (Erlanger, Kokowsky, & Cohen, 1961). The thermal stability of TR and nanoparticles-TR complex was determined by incubation for 20 min at given temperature. After the incubation the preparation was left for 1 h at room temperature before enzyme activity was determined. The activity obtained at 37 °C was assigned a value of 100%.

2.7. Statistical analysis

The assays were performed at least in triplicate on separate occasions. The data collected in this study are expressed as the mean value ± standard deviation.

3. Results and discussion

3.1. Characterization of nanoparticles

Linoleic acid was covalently coupled to an amino group of chitosan using EDC, a water-soluble carbodiimide. Conjugation was confirmed by infrared spectrum and ^{1}H NMR (Chen et al., 2003). Fig. 1 shows the size distribution of the nanoparticles formed by LA-chitosan (0.5 g/L) in the PBS buffer after sonication. There are two peaks in the histogram, the majority number of the particles were around to 500 nm in size. The small numbers of large particles (~800 nm) were believed to be formed by aggregation between the small particles. The AFM image of the nanoparticles is shown in Fig. 2. The well-formed nanoparticles with structural integrity formed by LA-chitosan molecules during the sonication.

3.2. Stability of the particles

In our previous study (Liu et al., 2005), fluorescence data showed that LA modified-chitosan is able to form nanoparticles, which have hydrophobic microdomains at the concentration large than 5×10^{-2} mg/mL. These hydrophobic domains play a role of junction zones, which link multiple polymer molecules together in an intermolecular aggregate (Philippova, et al., 2001). Fig. 3 show that the particle size slightly increases with the decreasing of concentration, indicating that stable particles were preserved with a few loosening of initial particles and hydrophobic chains act as crosslinker to stabilize particles in this concentration range. Fig. 4 shows the particles size against the concentration of urea. The particles size increased with an increase in urea concentration. Urea can produce twofold effects: firstly, urea can break the intramolecular hydrogen bond and make the chitosan molecular

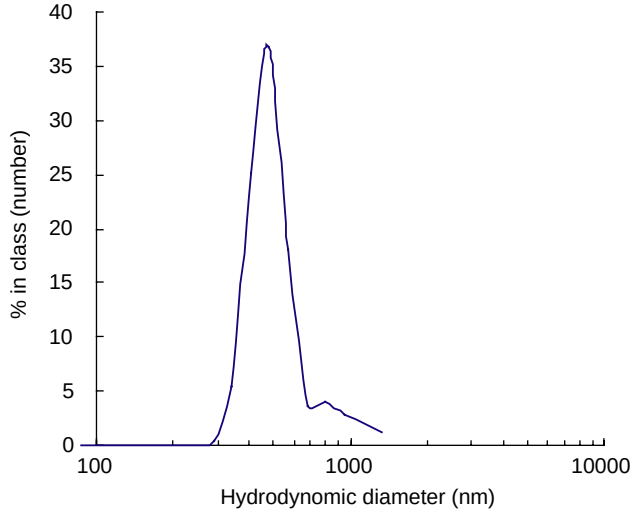

Fig. 1. Distribution of LA-chitosan nanoparticles in number ($\theta=90°$; $\lambda=670$ nm $T=25$ °C).

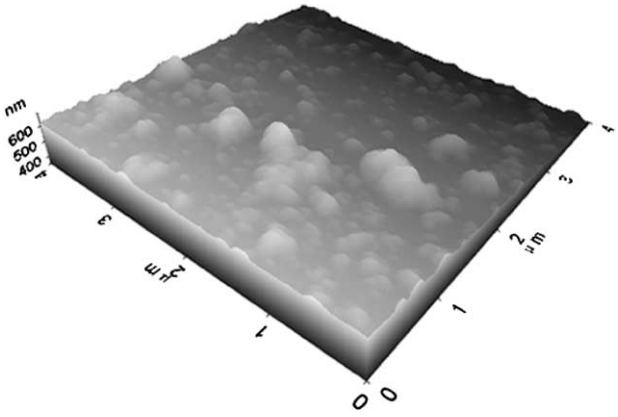

Fig. 2. Atomic force micrograph (AFM) of the naoparticles of LA-chitosan.

exist in extended form (Tsaih & Chen, 1997). Secondly, urea can severely disturb the hydrophobic interactions (Mukerjee & Ray, 1963). Philippova et al. (2001) found that hydrophobic modified chitosan has two kinds of hydrophobic domains: (1) the domains inherent to hydrophobic side chains of hydrophobic modified chitosan and (2) the domains inherent to chitosan itself. High concentration urea can destroy the first type of these domains. As a result, both these effects induce to form a loose sphere of large diameters.

Fig. 5 shows the particle size against NaCl concentration. Compared to salt-free solution, the particle size decreased 11.3% when NaCl concentration was 0.15 M. However, when high NaCl concentration (from 0.15 to 0.6 M) was applied, the size of particle was leveled off. The pH shift also affected the nanoparticle size. Fig. 6 shows that the particle size was larger in acidic solution than in neutral and basic solution. When low molecular weight salt was added in nanoparticle solution, the concentration of counter-ions rose and the shielding effect on the protonated amine group increased. Furthermore, the conformation of the chitosan becomes contracted and lead to a small size compacted sphere. On the other hand, in low pH solution the enforcing effect on the protonated amine group increase,

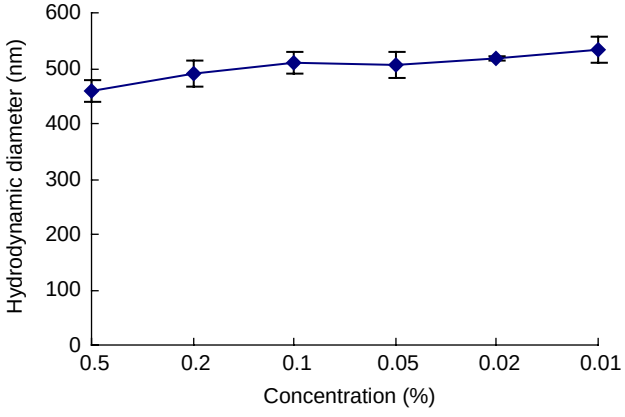

Fig. 3. The diameter of nanoparticles as a function of concentration of nanopaticle solution.

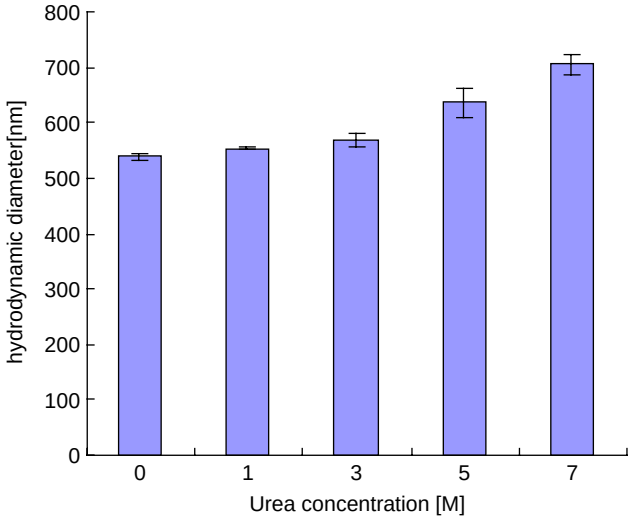

Fig. 4. Effect of concentration of urea on the diameter of nanoparticles.

and the electrostatic repulsion between the chitosan molecules also increase, which makes the chitosan molecule exist in extended conformation and lead to big size nanoparticles. (Chen, Lin, & Yang, 1994)

3.3. Adsorption of trypsin (TR)

The loading efficiency of TR was affected by its concentration and obtained results are presented in Fig. 7. As the concentration of TR increase 0.1–1 mg/mL, the protein loading capacity enhanced from 13.6±2.2 to 35.8±1.2%. But, the protein loading did not increase significantly at 2 mg/mL indicating that nanoparticles were almost saturated by TR at 1 mg/mL.

Urea is also valuable in studying protein adsorption phenomena (Poon, Clarke, & Schultz, 2001). Fig. 8 show

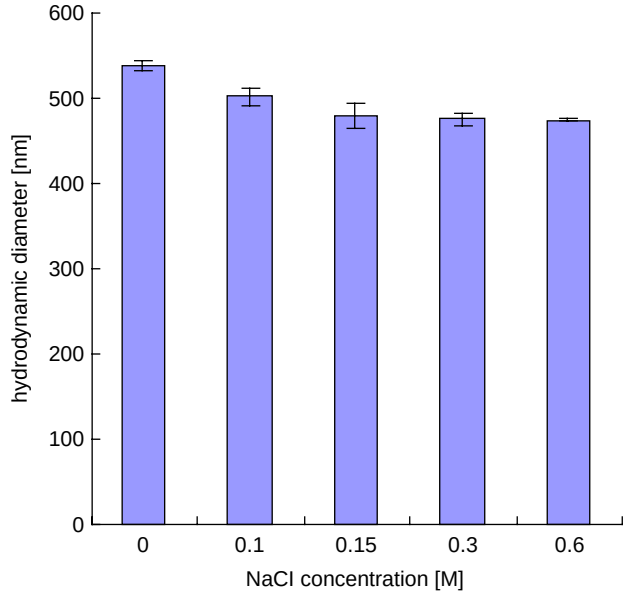

Fig. 5. Effect of concentration of NaCl on the diameter of nanoparticles.

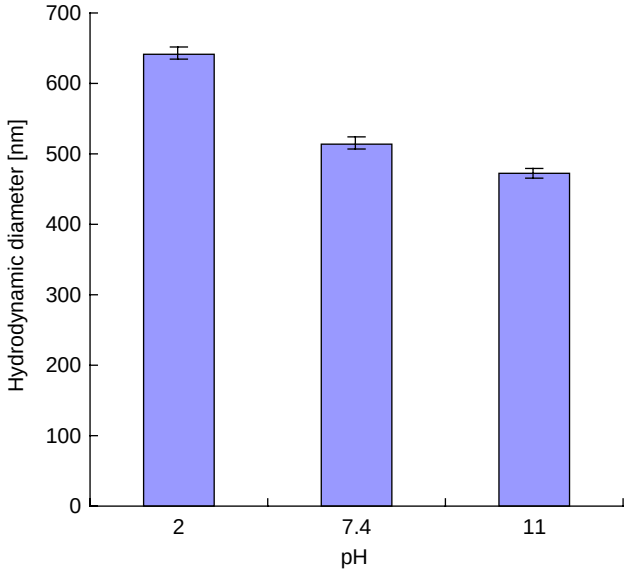

Fig. 6. Effect of pH on the diameter of nanoparticles.

that the loading capacity (LC) decreased as the concentration of urea increased, indicating urea may also disrupt the hydrogen bond between the TR and nanoparticles and reduce the driving force for adsorption TR hydrophobic groups to the nanoparticles. Basically, the electrostatic interaction in the core of nanoparticle might have been shielded by the increased NaCl concentration, thus led to dissociation. Fig. 9 shows that the binding of TR to nanoparticle was slightly influenced by NaCl concentration, indicating the presence of electrostatic interaction between nanoparticles and TR molecules. Itoyama and Tokura, (1994) found that compared to ionic strength and concentration of urea, pH shift significantly affect the hydrogen bonding between the protein (lysozyme) molecule and amide bonds on the carboxylated chitosan beads surface. In this study, there was remarkable influence by pH shift on the binding of TR to the LA-chitosan

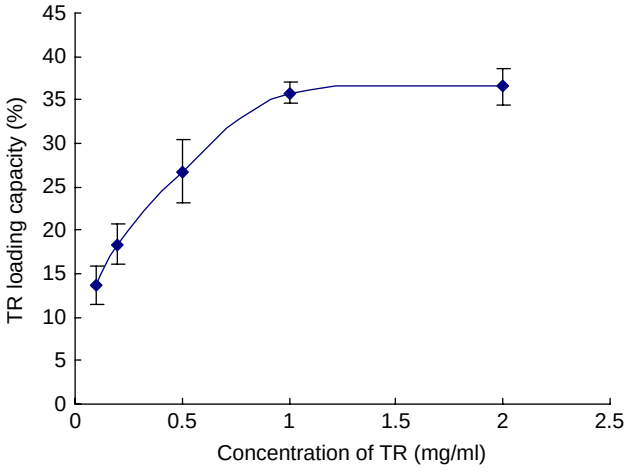

Fig. 7. The influence of TR concentration on loading capacity.

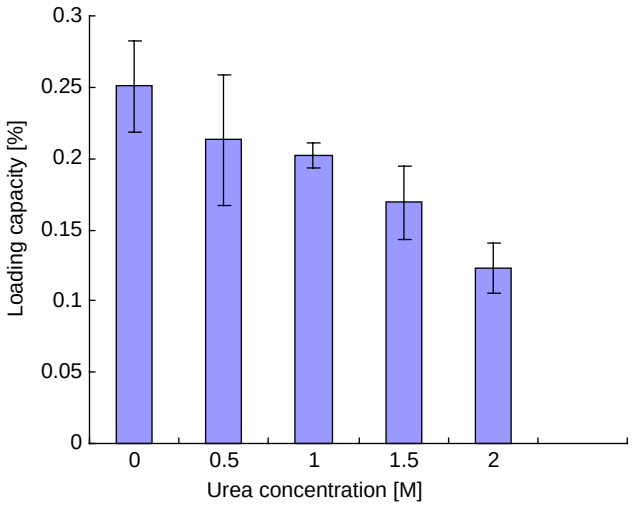

Fig. 8. Effect of concentration of urea on adsorption of trypsin to nanoparticles.

nanoaprticles (Fig. 10). It indicated that the main factors to regulate the TR adsorption on the particles is likely to be the hydrogen bonding between TR molecule and amide bonds on the nanoparticles surface.

3.4. Thermal stability of TR loading on nanoparticle

Protein unfolding causes denaturation at the higher temperature. Nishikawa, Akiyoshi, and Sunamoto (1994) firstly reported that the thermal denaturation of protein was drastically prevented upon the complexation with self-aggregate of cholesterol-bearing pullulan. The nanoparticles-TR complex was exposed to different incubation temperatures for 20 min. The activity of TR molecules on nanoparticles was found to have higher thermostability than that of free TR in solution (Fig. 11). Indeed, the former retained 72.8% of its activity after 20 min incubation at 80 °C, whereas free TR retained only 36.9% under the same

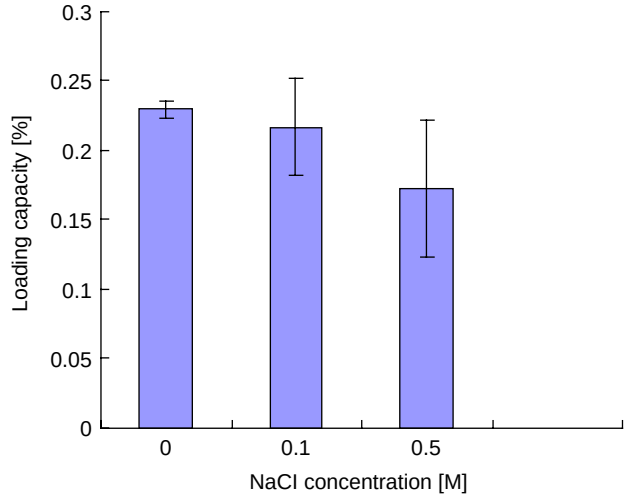

Fig. 9. Effect of concentration of NaCl on adsorption of trypsin.

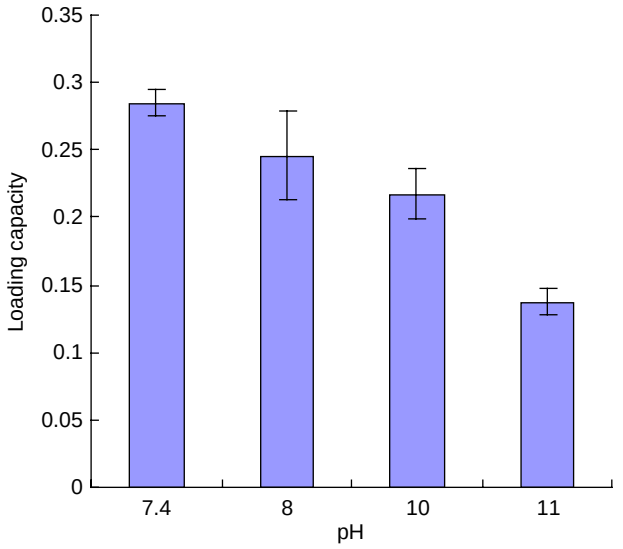

Fig. 10. Effect of pH on adsorption of trypsin.

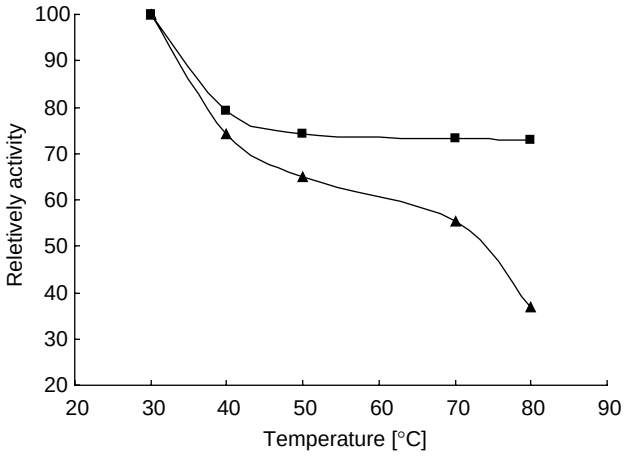

Fig. 11. Temperature dependence of thermal stability of free enzyme and immobilized TR. The free enzyme and immobilized TR were preincubated in PBS buffer 7.4 at different temperature for 20 min intervals and assayed under standard conditions. Symbol: (▲) free enzyme; (■) immobilized TR.

conditions. The improve stability for nanoparticle-coupled enzyme is likely due to the increased conformation stability upon immobilization. According to the above information, the multi-interaction (e.g. electrostatic interaction, hydrogen bonding, and hydrophobic interaction) between the TR and the nanoparticle must be the principal driving force of the stabilization.

4. Conclusion

Addition of salt, urea or variations of pH can slightly affect the size of nanoparticles with structural integrity. The TR can be loaded on nanoparticles and the mechanism has been suggested to be regulated by several factors such as

hydrogen bonding, hydrophobic interactions and ionic interactions, which must be responsible for stabilization of loaded TR. These results suggest that the nanoparticles based on linoleic-acid modified chitosan can be useful as a carrier for biomolecules in the fields of biotechnology and pharmaceutical sciences.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R & D Project (A050376), Ministry of Health & Welfare, Republic of Korea.

References

Akiyoshi, K. (2002). Hydrophobic effects. In N. Yui (Ed.), *Supermolecular design for biological applications* (pp. 13–24). Boca Raton: CRC Press.

Akiyoshi, K., Kobayashi, S., Shichibe, S., Mix, D., Baudys, M., Kim, S. W., et al. (1998). Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: Complexation and stabilization of insulin. *Journal of Controlled Release*, *54*, 313–320.

Akiyoshi, K., & Sunamoto, J. (1996). Supramolecular assembly hydrophobized polysaccharides. *Superamolecular Science*, *3*, 157–163.

Arvanitoyannis, I. (1999). Totally-and-partially biodegradable polymer blends based on natural and synthetic macromolecules: Preparation and physical properties and potential. *Journal of Macromolecular Science-Review Macromolecular Chemistry. Physics C*, *39*, 205–271.

Arvanitoyannis, I., Nakayama, A., & Aiba, S. (1998). Chitosan and gelatine based edible films: State diagrams, mechanical and permeation properties. *Carbohydrate Polymer*, *37*, 371–382.

Calvo, P., Remunan-Lopez, C., Vila-Jato, J. L., & Alonso, M. J. (1997). Novel hydrophilic chitosan–polyethylene oxide nanoparticles as protein carriers. *Journal Applied Polymer Science*, *63*, 125–132.

Chen, R. H., Lin, J. H., & Yang, M. H. (1994). Relationships between the chain flexibilities of chitosan molecules and the physical properties of their casted films. *Carbohydrate Polymer*, *241*, 41–46.

Chen, X. G., Lee, C. M., & Park, H. J. (2003). O/W emulsification for the self-aggregation and nanoparticle formation of linoleic acid-modified chitosan in the aqueous system. *Journal of Agriculture Food Chemistry*, *51*, 3135–3139.

Chung, H., Kim, T. W., Kwon, M., Kwon, I. C., & Jeong, S. Y. (2001). Oil components modulate physical characteristics and function of the natural oil emulsions as drug or gene delivery system. *Journal of Controlled Release*, *71*, 339–350.

Erlanger, B. F., Kokowsky, N., & Cohen, N. (1961). The preparation and properties of two new chromogenic substrate of trypsin. *Archives of Biochemistry and Biophysics*, *95*, 271–278.

Harada, A., & Kataoka, K. (1998). Novel polyion complex micelles entrapping enzyme molecules in the core: Preparation of narrowly-distributed micelles from lysozyme and poly (ethylene glycol)-poly (aspartic acid) block copolymer in aqueous medium. *Macromolecule*, *31*, 288–294.

Harada, A., & Kataoka, K. (2001). Pronounced activity of enzymes through the incorporation into the core of polyion complex micelles made from charged block copolymers. *Journal of Controlled Release*, *72*, 85–91.

Itoyama, K., & Tokura, S. (1994). Adsorption behavior of lysozyme onto carboxylated chitosan beads. *Sen-i Gakkaishi*, *50*, 118–123.

Jaturanpinyo, M., Harada, A., Yuan, X., & Kataoka, K. (2004). Preparation of bionanoreactor based on core–shell structured polyion complex micelles entrapping trypsin in the core cross-linked with glutaraldehyde. *Bioconjugate Chemistry*, *15*, 344–348.

Kim, Y. H., Gihm, S. H., & Park, C. R. (2001). Structural characteristics of size-contrlled self-aggreegates of deoxycholic acid-modified chitosan and their application as a DNA delivery carrier. *Bioconjugate Chemistry*, *12*, 932–938.

Kwon, S., Park, J. H., Chung, H., Kwon, I. C., Jeong, S. Y., & Kim, I. S. (2003). Physicochemical characteristics of self-assembled nanoparticles based on glycol chitosan bearing 5β-cholanic acid. *Langmuir*, *19*, 10188–10193.

Lee, K. Y., Jo, W. H., Kwon, I. C., Kim, Y.-H., & Jeong, S. Y. (1998a). Physicochemical characteristics of self-aggregates of hydrophobically modified chitosans. *Langmuir*, *14*, 2329–2332.

Lee, K. Y., Jo, W. H., Kwon, I. C., Kim, Y. H., & Jeong, S. Y. (1998b). Structural determination and interior polarity of self-aggregates prepared from deoxycholic acid-modified chitosan in water. *Macromolecule*, *31*, 378–383.

Lee, K. Y., Kim, Y. H., Kwon, I. C., Jo, W. H., & Jeong, S. Y. (2000). Self-aggregates of deoxycholic acid-modified chitosan as a novel carrier of adriamycin. *Colloid and Polymer Science*, *278*, 1216–1219.

Liu, C. G., Desai, K. G., Chen, X. G., & Park H. J. (2005). Linoleic-acid modified chitosan for formation of self-assembled nanoparticles. *Journal of Agriculture Food Chemistry*, 53, 437–441.

Liu, W. G., Zhang, X., Sun, S. J., Sun, G. J., & Yao, K. D. (2003). N-alkylated chitosan as a potential nonviral vector for gene transfection. *Bioconjugate Chemistry*, *14*, 782–789.

Mukerjee, P., & Ray, A. (1963). The effect of urea on micelle formation and hydrophobic bonding. *Journal of Physical Chemistry*, *67*, 190–192.

Nishikawa, T., Akiyoshi, K., & Sunamoto, J. (1994). Superamolecular assembly between nanoparticles of hydrophobized polysaccharide and soluble protein complexation between the self-aggregate of cholesterol-bearing pullulan andα-chymotrypsin. *Macromolecules*, *27*, 7654–7659.

Nishikawa, T., Akiyoshi, K., & Sunamoto, J. (1996). Macromolecular complexation between bovine serum albumin and the self-assembled hydrogel nanoparticle of hydrophobized polysaccharides. *Journal of American Chemistry Society*, *118*, 6110–6115.

Park, J. H., Kwon, S., Nam, J. O., Park, R. W., Chung, H., Seo, S. B., et al. (2004). Self-assembled nanoparticles based on glycol chitosan bearing 5β-cholanic acid for RGD peptide delivery. *Journal of Controlled Release*, *95*, 579–588.

Philippova, O. E., Volkov, E. V., Sitnikova, N. L., Khokhlov, A. R., Desbrieres, J., & Rinaudo, M. (2001). Two types of hydrophobic aggregates in aqueous solutions of chitosan and its hydrophobic derivative. *Biomacromolecules*, *2*, 483–490.

Poon, S., Clarke, A. E., & Schultz, C. J. (2001). Effect of denaturants on the emulsifying activity of proteins. *Journal of Agriculture and Food Chemistry*, *49*, 281–286.

Sashiwa, H., & Aiba, S. I. (2004). Chemically modified chitin and chitosan as biomaterials. *Progress Polymer Science*, *29*, 887–908.

Sashiwa, H., Fujishima, S., & Yamano, N. (2003). Enzymatic production of *N*-acetyl-D-glucosamine from chitin. Degradation study of *N*-acetylchitooligosaccharide and the effect of mixing of crude enzymes. *Carbohydrate Polymer*, *51*, 391–395.

Tsaih, M. L., & Chen, R. H. (1997). Effect of molecular weight and urea on the conformation of chitosan molecules in dilute solutions. *International Journal of Biological Macromolecules*, *20*, 233–240.