Preparation, characterization and evaluation of antibacterial activity of catechins and catechins–Zn complex loaded β-chitosan nanoparticles of different particle sizes

Hongcai Zhang a, b, Jooyeoun Jung a, Yanyun Zhao a, * 

a Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331–6602, USA 
b School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, PR China

A R T I C L E   I N F O

Article history:
Received 5 August 2015
Received in revised form 28 September 2015
Accepted 10 October 2015
Available online 22 October 2015

Keywords:
β-Chitosan nanoparticles 
Catechins–Zn complex 
Encapsulation 
Stability 
Structures 
Antibacterial activity

A B S T R A C T

This study used β-chitosan nanoparticles (β-CS NPs) of different particle sizes to encapsulate catechins (CAT) or CAT–Zn complex by ionic gelation technology. The antibacterial activity of CAT or CAT–Zn complex loaded β-CS NPs against Escherichia coli and Listeria innocua were investigated based on bacterial growth curve, minimum inhibitory concentration (MIC), and minimum bacterial concentration (MBC). Fourier transform infrared spectrometer (FT-IR) was employed to study the incorporation of CAT or CAT–Zn complex into β-CS NPs. The CAT–Zn complex loaded β-CS NPs had particle size of 208–591 nm, polydispersity index (PDI) of 0.377–0.395, and positive Zeta-potential of 39.17–45.62 mV. The CAT–Zn complex loaded β-CS NPs of smaller particle sizes showed higher antibacterial activity than that of larger particle size ones. The MIC and MBC of CAT–Zn complex loaded β-CS NPs of the smallest particle size against L. innocua and E. coli were 0.031 and 0.063 mg/mL, and 0.063 and 0.125 mg/mL, respectively. This study suggested that encapsulation of CAT–Zn complex in β-CS NPs improved the antibacterial activity of CAT and CAT–Zn complex, and the encapsulators have great potential to be used as antibacterial substances for food and other applications through either direct addition or incorporation into packaging materials.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Beta-chitosan (CS) is originated from the deacetylation of β-chitin that exists mainly in the squids pens. Along with increased production of squids, β-CS has attracted increasing attention due to its unique functionalities, including simpler preparation, higher affinity toward solvents, and better biological activity than α-CS from crab and shrimp shells (Jung & Zhao, 2011; Subhapradha et al., 2013). Meanwhile, nanotechnology is continuously found its ways in various fields, including food processing and packaging, biomedicine, and pharmacy for enhancing process performance and product functionality (Chen, Remondetto, & Subirade, 2006; Kataoka, Harada, & Nagasaki, 2012; Ezhilarasi, Karthik, Chhanwal, & Anandharamakrishnan, 2013). Nanoparticles (NPs) provide a promising mean to encapsulate chemically labile bioactive compounds for improving their stability during food processing and storage and in the gastrointestinal tract (Chen & Subirade, 2005), and to enhance the bioavailability of poorly soluble bioactive compounds (Matalanis, Jones, & McClements, 2011).

Chitosan nanoparticles (CS NPs) have been employed to encapsulate bioactive compounds, such as polyphenols through different approaches, including ionic gelation (Zhang & Zhao, 2015), spray drying (Ersus & Yurdagel, 2007), coacervation (Shutava, Balkundi, & Lvov, 2009), liposome entrapment (Takahashi et al., 2007), inclusion complexation (Mercader-Ros, Lucas-Abellán, Fortea, Gabaldón, & Núñez-Delgado, 2010), cocrystallization (Deladino, Anbinder, Navarro, & Martino, 2007), nanoencapsulation (Barras et al., 2009), freeze drying (Laine, Kylli, Heinonen, & Jouppila, 2008), yeast encapsulation (Shi et al., 2007), and emulsion (McClements, Decker, Park, & Weiss, 2009). Among them, ionic gelation using sodium tripolyphosphate (TPP) was preferred because the sodium TPP has quick gelling capability and is safe to use (Fan, Yan, Xu, & Ni, 2012). The CS and sodium TPP formed NPs are typically stabilized through electrostatic crosslinks between the positively charged amino groups in CS molecules and the negatively charged sodium TPP.

Catechins (CAT) as a group of polyphenolic compounds are widely used as nutraceuticals for improving antioxidant,
anti-inflammatory, and antibacterial activities ( Bennick, 2002 ;
Chung et al., 2000 ). However, CAT has low utilization efficacy, and
only a small proportion of the molecules remains available follow-
ing oral administration, due to insufficient gastric residence time,
low permeability and/or solubility within the gut, as well as their
instability in the gastrointestinal tract ( low pH , enzyme activity,
presence of other nutrients and other interfering agents ). Previ-
ous studies have reported that tea polyphenol–Zn complex (the
analogue of CAT–Zn complex ) can change the distribution of elec-
tron cloud of tea polyphenol and has higher bioactivity than that
of tea polyphenol alone since the aromatic hydroxyl group in tea
polyphenol was activated after Zn and tea polyphenol were com-
bined ( Wang, Zhang, Liu, Liu, & Yang, 2012 ; Zhang & Zhao, 2015 ).
Hence, it was hypothesized that the encapsulation of CAT or CAT–Zn
complex can protect them from adverse environmental attack, such
as light, moisture, and oxygen, in turn enhance their biological
activities ( i.e. , antioxidant and antibacterial activities ). Although
previous study had investigated the encapsulation of CAT using
starch and their derivatives, such as amylase, amylopectin, dex-
trin, polydextrose and cellulose ( Gadkaria & Balaramana, 2015 ),
no study had evaluated the encapsulation of CAT or CAT–Zn com-
plex using β-CS NPs. CS NPs have high encapsulation efficacy and
can control the releasing rate of encapsulated bioactive compounds
( Fang & Bhandari, 2010 ). Moreover, the particle size of the encap-
sulators directly impacts their performance. In general, the small
size of particles penetrates more effectively into the target cells,
thus improving the encapsulation efficacy of target substances
( Dziuzak, 1998 ). Therefore, to better understand the antibacterial
mechanisms of CAT or CAT–Zn complex loaded β-CS NPs,
different particle sizes of β-CS NPs were synthesized and their antibacterial activity was investigated accordingly in this
study. Additionally, the CS NPs are very sensitive to the pH envi-
nronment of the preparation solutions, and are easily to precipitate
during storage at room temperature. Hence, it is important to
study the effects of different pH of β-CS NPs solutions on the par-
ticle size of β-CS NPs and different pH of β-CS NPs solutions on
the stability (precipitation) of CAT and CAT–Zn complex loaded
β-CS NPs.

The objectives of this study were to (1) investigate the parti-
cle size and polydispersity index (PDI) of CAT or CAT–Zn complex
loaded β-CS NPs prepared at different ratios of CS and CAT
or CAT–Zn complex, (2) characterize the structures of CAT or CAT–Zn
complex loaded β-CS NPs using Fourier transform infrared spec-
rometer (FT-IR), (3) determine the effect of different pH of β-CS
NPs solutions on the stability of CAT and CAT–Zn complex loaded
β-CS NPs, and (4) study the antibacterial activity (strains growth
curve, minimum inhibitory concentration (MIC), and minimum
bacterial concentration (MBC)) of CAT or CAT–Zn complex loaded
β-CS NPs at different particle sizes.

2. Materials and methods

2.1. Materials and reagents

Beta-chitin and CS were prepared in our laboratory through
deproteinization of jumbo squid pens ( Dosidicus gigas ) using the
procedures from Jung and Zhao ( 2011 ). The Whatman® cellulose
nitrate membrane filters (0.45 and 0.25 μm) were from What-
man GmbH (Dassel, Germany). Cellulase was obtained from Tokyo
Chemical Industry, Co. Ltd. (Japan), CAT and sodium TPP were
purchased from Sigma Chemical Co. (St. Louis, MO). Other analytical
grade reagents including acetic acid, sodium hydroxide, hydroclo-
rnic acid, and zinc acetate were from Alfa Aesar, A Johnson Matthey
Company (MA, USA). Distilled (DI) water was used throughout this
study.

2.2. Preparation of low molecular weight (MM) of β-CS

For obtaining small particle size of CS NPs, it is necessary to start
with low MM of CS ( Zhang & Zhao, 2015 ). To prepare low MM of β-
CS, β-chitin obtained from squid pens were ground, deproteinize
by treating in 50% NaOH for 9 h at 80 °C, washed with DI water,
and then dried at 40 °C oven for 24 h to obtain β-CS ( Jung & Zhao,
2011 ). The obtained β-CS (with original MM of 3,500 kDa) was fur-
ther depolymerized using cellulase to obtain small MM of β-CS for
preparing nanoparticles. Briefly, 2.5 g of β-CS was dissolved in 1%
acetic acid solution and stirred overnight, and then 2.5% (w/w) cel-
lulase was added for reacting up to 3 h. The 4 N NaOH was further
added to reach a final pH 11 for precipitation. The hydrolyzates
were boiled for 10 min to inactivate enzyme and centrifuged at
8,000 × g for 30 min to remove denatured enzyme (Sorval Cen-
trifuges, Dupont Co., Wilmington, Delaware, USA). The precipitated
samples were washed using DI water until pH reached neutral for
removing denatured enzyme and other residues, and were then
dried for later use. The degree of deacetylation (DDA) and MM of
β-CS were ~86% and ~160 kDa, respectively, determined using the
methods from Jung and Zhao ( 2011 ).

2.3. Preparation of β-CS NPs and CAT or CAT–Zn complex loaded
β-CS NPs at different particle sizes

Beta-CS NPs were prepared following the procedures described
in our previous study with slight modification ( Zhang & Zhao,
2015 ). Briefly, β-CS was dissolved in aqueous acetic acid
(0.1 mg/ml) at a concentration of 0.25 mg/ml, stirred overnight,
and filtered through a 0.45 μm filter membrane. The pH of the
resulting solutions was adjusted to 4.7–4.8 by adding 4% NaOH.
Sodium TPP was dissolved in DI water at a concentration of
0.25 mg/ml (pH: 7.0) and passed through 0.25 μm filter membrane.
To prepare β-CS NPs with different particle sizes, 15 ml of β-CS
solution was placed in a 100 ml round-bottom flask, and then dif-
ferent amounts of sodium TPP at β-CS to sodium TPP solution ratios
of 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1 (w/w) were added into the above
β-CS solution under stirring at 600 rpm by fixing the concentra-
tion of β-CS ( Zhang & Zhao, 2015 ). The reaction was carried out for
30 min and the resulting suspension was subjected to centrifuga-

tion for collecting the sediments for further analysis. To investi-
gate the effect of different pH of β-CS solution on the particle size,
PDI, and Zeta-potential of β-CS NP dispersion, the pH of β-CS solution
was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 using 0.1 N NaOH or
HCl.

To obtain CAT and CAT–Zn complex loaded β-CS NPs at differ-
ent particle sizes, 15 ml of β-CS solution was placed in a 100 ml
round-bottom flask and stirred at 600 rpm, and then CAT or CAT–Zn
complex at the ratios of 1:1, 1:3 and 1:5 (w/w β-CS) were added
into the above β-CS solution under stirring, followed with quick
addition of 3 ml of sodium TPP solution. The reaction was car-
ried out for 30 min and the resulting suspension was subjected to
centrifugation for collecting the sediments for further analysis.

2.4. Determination of particle size, PDI, Zeta-potential, and
encapsulation efficacy of CAT and CAT–Zn complex loaded β-CS
NPs

The β-CS NPs solution and CAT and CAT–Zn complex loaded
β-CS NPs solutions were centrifuged at 12,000 rpm for 1 h. The
obtained sediments were re-suspended in DI water, subjected to
ultrasound treatment for 20 min before use, and further character-
ized in terms of their particle size, particle size distribution, and
Zeta-potential using dynamic light scattering (DLS) particle size
analyzer (DB-525, Brookhaven Instrument Corporation, Holtsville,
NY, USA) ( Zhang & Zhao, 2015 ). To determine the encapsulation...
efficacy of CAT and CAT–Zn complex and the yield of NPs, the sediment of CAT and CAT–Zn complex loaded β-CS NPs were collected by centrifugation at 12,000 rpm for 1 h, and a small amount of glycerol was added to avoid the aggregation of NPs before freeze-dried (Consol 4.5, The Virtis Company Inc., Gardiner, NY, USA). The freeze-dried samples were then calculated for the yield. The encapsulation efficacy of CAT was directly measured using UV/vis spectrophotometer at 275 nm (UV−1.800 UV−vis Spectrophotometer Shimadzu Corporation, Japan). To determine the encapsulation efficacy of CAT–Zn complex, Zn was first removed from the CAT–Zn complex solution. For doing it, 5 mL of CAT−Zn complex loaded β-CS NPs solution was dissolved in 40 mL of 0.1 M HCl solution, and Zn was extracted from above solution using ultrasound treatment for 5 min (Model B−220H, Branson Cleaning Equipment Company, Parrott Drive, Shelton, Conn, USA), and above mixture solution was then layered using 40 mL of acetic ether twice. The solution after layering was evaporated by a rotary evaporator (Buchi Rotary evaporator B−124, Stanwood, Washington, USA) and obtained segments were redissolved in DI water in a 25 mL of volumetric flask for determining CAT content.

Encapsulation efficacy (%) = \[ \frac{\text{Mass of CAT or CAT−Zn complex-added} - \text{mass of unencapsulated CAT or CAT−Zn complex}}{\text{Amount of CAT or CAT−Zn complex added}} \times 100 \] (1)

The yield was calculated as

NPs yield (%) = \[ \frac{\text{Mass of recovered nanoparticles}}{\text{Total amount of sodium TPP, CS and CAT or CAT−Zn complex added}} \times 100 \] (2)

2.5. Stability of freshly prepared β-CS NPs solutions and CAT or CAT–Zn complex loaded β-CS NPs at different pH

Among many interference factors to the stability of NPs solutions, pH is very important and directly affects the precipitation of β-CS NPs solution. Therefore, the pH of freshly prepared β-CS NPs solution and CAT and CAT−Zn complex loaded β-CS NPs solutions were adjusted to 1.2, 2.5, 6.0, 6.6 and 7.4 by 0.1 N NaOH or HCl to investigate the stability of β-CS NPs solutions and CAT or CAT−Zn complex loaded β-CS NPs solutions at these different pH environments. The dispersion stabilities of the sample solutions were evaluated by using the turbidity of the solutions (light transmittance) using a UV−vis spectrophotometer at 500 nm.

2.6. FT-IR analysis

All samples were smashed completely (Model 3383L10, Thomas Wiley® Mini-Mill, Thomas Scientific Corporation, New Jersey, USA) and 10 mg of samples was analyzed as KBr pellets (1:99, w/w) using a Nicolet i550 FT-IR (Thermo Scientific, Madison, WI). Spectral scanning was taken in the wavelength range between 500 and 4,000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) with scan speed of 2 mm/s.

2.7. Antibacterial analysis

2.7.1. Determination of bacterial growth rate

For determining bacterial growth rate, two non-pathogenic bacterial strains including Gram-negative strain Escherichia coli (ATCC 25922) and Gram-positive strain Listeria innocua (ATCC 51742) were cultured in Tryptic soy broth and BactoTM Brain Heart infusion, respectively. The strain suspension was cultured in an incubator (Lab-Line Orbit shaker bath model 3527, Melrose Park, Illinois, USA) at 37°C and taken out at various time intervals. Tryptic soy broth and BactoTM Brain Heart infusion without sample solutions were used as the negative control. Bacterial solution (10\(^7\) CFU/mL) was used as positive control. Bacterial growth rates in the presence of CAT and CAT−Zn complex loaded β-CS NPs at different particle sizes were determined by measuring the optical density at 600 nm (OD\(_{600}\)) using the same UV−vis spectrophotometer based on the turbidity of the cell suspension.

2.7.2. Inhibition zone of β-CS NPs and CAT or CAT−Zn complex loaded β-CS NPs

The disc diffusion method was employed to determine the inhibition zone of β-CS NPs and CAT or CAT−Zn complex loaded β-CS NPs (Qi, Xu, Jiang, Hu, & Zou, 2004). A 100 µL of suspension containing 10\(^5\) CFU mL/L of each bacterial strain was spread on the DifcoTM Brain Heart infusion agar and Tryptic soy agar, respectively. The filter papers were cut into 6 mm diameter disc, sterilized at 121°C for 15 min (Model SR-24A-ADVPB, Consolidated, Machine Corporation, Boston, MA, USA), dried in an oven at 37°C for 1 h, and was then placed on the petri dish containing the DifcoTM Brain Heart infusion agar and Tryptic soy agar. A 10 µL of β-CS NPs solution or CAT or CAT−Zn complex loaded β-CS NPs solutions (1 mg/mL, pH 4.5) were dropwise added into the disc. The antibacterial activity of β-

CS NPs and CAT or CAT−Zn complex loaded β-CS NPs was evaluated by measuring the zone of inhibition against the test bacteria.

2.7.3. Measurement of minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC)

MIC and MBC of β-CS NPs and CAT or CAT−Zn complex loaded β-CS NPs at different particle sizes were determined using the turbidimetric and micro-dilution methods, respectively (Qi et al., 2004). About 10\(^7\) CFU/mL of E. coli and L. innocua were suspended in sterile BactoTM Brain Heart infusion and Tryptic soy broth, respectively, in which different concentrations of each sample solution was added. A series of test tubes each containing 5.0 mL of culture medium were autoclaved at 121°C for 15 min. The β-CS, β-CS NPs powders and CAT or CAT−Zn complex loaded β-CS NPs powders at different particle sizes were accurately quantified and added into DI water, the pH of all sample solutions was adjusted to 4.5 using 0.25% acetic acid. For the first tube, 5.0 mL of β-CS solution, β-CS NPs solution or CAT or CAT−Zn complex loaded β-CS NPs solutions (1 mg/mL) was added, respectively. After mixing, 5.0 mL of the mixture was transferred to the second tube, and similar transformation was repeated. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The tubes were inoculated with 50 µL of freshly prepared bacteria suspension under aseptic conditions. All samples were adjusted to pH 4.5 with 0.1 M NaOH for equal comparisons and incubated at 35°C for 24 h to evaluate MIC subsequently. MBC is defined as the concentration producing a 99.9% reduction of colony number in the initial inoculum by assaying the live organisms in those tubes from the MIC test that showed no growth. A 200 µL of each strain solution from each of those tubes were inoculated on DifcoTM Brain Heart infusion agar and Tryptic soy agar, respectively, and examined for the signs of growth. Growth of bacteria demonstrated the presence of the bacteria in the original MIC tube. If no growth was observed, the original tube contained no living bacteria.
Table 1

<table>
<thead>
<tr>
<th>Ratios of chitosan and sodium TPP</th>
<th>Polydispersity index (PDI)</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:1</td>
<td>0.253 ± 0.017</td>
<td>15.58 ± 0.12</td>
</tr>
<tr>
<td>4:1</td>
<td>0.273 ± 0.011</td>
<td>24.51 ± 0.42</td>
</tr>
<tr>
<td>5:1</td>
<td>0.317 ± 0.010</td>
<td>27.25 ± 1.06</td>
</tr>
<tr>
<td>6:1</td>
<td>0.344 ± 0.006</td>
<td>26.57 ± 0.80</td>
</tr>
<tr>
<td>7:1</td>
<td>0.363 ± 0.04</td>
<td>28.53 ± 0.33</td>
</tr>
<tr>
<td>8:1</td>
<td>0.333 ± 0.033</td>
<td>31.89 ± 2.79</td>
</tr>
</tbody>
</table>

PH of β-chitosan nanoparticle solutions

<table>
<thead>
<tr>
<th>pH</th>
<th>Polydispersity index</th>
<th>Zeta-potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>0.300 ± 0.027</td>
<td>13.67 ± 1.88</td>
</tr>
<tr>
<td>4.0</td>
<td>0.197 ± 0.012</td>
<td>18.24 ± 0.67</td>
</tr>
<tr>
<td>5.0</td>
<td>0.337 ± 0.018</td>
<td>20.00 ± 1.25</td>
</tr>
<tr>
<td>6.0</td>
<td>0.317 ± 0.035</td>
<td>20.65 ± 1.14</td>
</tr>
<tr>
<td>7.0</td>
<td>0.134 ± 0.049</td>
<td>25.35 ± 1.74</td>
</tr>
<tr>
<td>8.0</td>
<td>0.196 ± 0.034</td>
<td>6.51 ± 0.89</td>
</tr>
</tbody>
</table>

1 pH of solution was 5.0.

2.8. Experimental design and statistical analysis

A completely randomized experimental design was applied by considering different particle sizes of CAT or CAT–Zn complex loaded β-CS NPs, which was achieved by varying the ratios of β-CS vs. CAT or CAT–Zn complex (w/w, 1:1, 1:3 and 1:5) in the solution mixtures. The analysis of variance (one-way ANOVA analysis) using SPSS program (SPSS 17.0, IBM SPSS Institute, Inc., New York, USA) was applied to analyze the experimental data of different treatment groups (T0, T1, T2, T3, T4, T5 and T6) using the Least Significant Difference (LSD) test and Student–Newman–Keuls test (S–N–K) with a significance level of P < 0.05.

3. Results and discussion

3.1. Optimized conditions for preparing small particle size of β-CS NPs

The physicochemical properties of NPs directly impact their physiological functions. Most importantly, the particle size of NPs affects the mucosal and epithelial tissue uptake, and intracellular trafficking (Panyam & Labhasetwar, 2003). Since subcellular size of NPs from 1 to 200 nm gives more surface areas available to the mucosa of small intestine and may extend the gastric residence time of bioactive compounds, reducing the particle size of NPs is important during the preparation of NPs (Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013). Moreover, surface charge determines not only the stability and permeation enhancing effects of the NPs (Smith, Woodh, & Dornish, 2004), but also the ability of NPs to escape from the endolysosomes (Panyam, Zhou, Prabha, Sahoo, & Labhasetwar, 2002). Hence, for producing small particle size of NPs and obtaining desirable Zeta-potential, β-CS was firstly degraded to small MM of ~160 kDa and the ratio of β-CS and sodium TPP were optimized for preparing β-CS NPs of small particle size. Particle size, PDI and Zeta-potential of β-CS NPs were prepared at the different ratios of β-CS and sodium TPP and at different pH of β-CS solution are reported in Table 1.

The smallest particle size and the corresponding PDI and Zeta-potential were 59.20 nm, 0.317, and 27.25 mV, respectively, obtained at a β-CS to sodium TPP ratio of 5:1 and pH 5.0 of sample preparation solution. The Zeta-potential of the β-CS NPs was increased along with increased concentration of β-CS, which was attributed to the increase of NH₃⁺ protonated groups of CS and the aggregation of CS NPs due to large surface area and high charge repulsion. PDI was also increased when β-CS concentration increased from 3:1 to 5:1, probably owning to the aggregation of NPs at small particle size. However, no further increase was observed after that, which might be explained as the redundant β-CS affected the particle size distribution of β-CS NPs at the higher β-CS to sodium TPP ratios, but the exact reasons were still unknown.

In respect to the pH effect, Zeta-potential of β-CS NPs generally showed an increased trend along with increased pH from 3 to 7. However, significant decrease in Zeta-potential was observed at pH 8, probably because of the deprotonation of β-CS at high pH and increased surface charge of β-CS NPs. The particle size and PDI of β-CS NPs showed no clear trend with the increase of pH. At pH 3.0–5.0, the particle size of β-CS NPs increased first and then decreased, but increased again at pH 5.0–7.0. This might be because the crosslinking force at low pH was much weaker than that at high pH. And precipitation of β-CS occurred after reaching pH 5.0, because the pKa of β-CS is between 5.5 and 6.5. The β-CS NPs reached the smallest particle size of 99.13 nm at pH 5.0 (Table 1). The PDI was in the range of 0.134–0.337 for all β-CS NPs. Considering the particle size and PDI of β-CS NPs comprehensively, the β-CS to sodium TPP ratio of 5:1 and pH 5.0 were selected as optimized conditions for preparing β-CS NPs.

3.2. Physicochemical characteristics of β-CS NPs and CAT or CAT–Zn complex loaded β-CS NPs at different particle sizes

Particle size distributions of CAT or CAT–Zn complex loaded β-CS NPs were illustrated in Fig. 1, and the particle size, PDI, Zeta-potential, yield and encapsulation efficacy of CAT or CAT–Zn complex loaded β-CS NPs are reported in Table 2.

The particle sizes of CAT–Zn complex loaded β-CS NPs were normally distributed (Fig. 1E, F and G) in a range of 208 to 590 nm (Table 2), higher than that of CAT loaded β-CS NPs under the same ratio of β-CS to sodium TPP (116 to 627 nm, Fig. 1B, C and D). This result was inconsistent with our previous report (Zhang & Zhao, 2015), in which the particle size of tea polyphenols–Zn complex loaded β-CS NPs was smaller than that of tea polyphenols loaded β-CS NPs. This difference might be attributed to the increased encapsulation of CAT–Zn complex loaded β-CS NPs, resulting in the aggregation of small size of particles. Moreover, the particle size of CAT or CAT–Zn complex loaded β-CS NPs were larger than those of β-CS NPs, suggesting that CAT or CAT–Zn complex had been incorporated into β-CS NPs. The PDI of different particle sizes of CAT or CAT–Zn complex loaded β-CS NPs had a wide range from 0.329 to 0.421 (Table 2), but all were less than 0.3, indicating good PDI of the NPs solution (Fan et al., 2012; Comfort, Maurer, & Hussain, 2014). The yields of CAT or CAT–Zn complex loaded β-CS NPs were all decreased with increased ratios of β-CS:CAT or CAT–Zn complex (1:1 to 1:5). Moreover, the yields of CAT loaded β-CS NPs were higher than that of CAT–Zn complex loaded β-CS NPs at the same conditions, probably owning to the high aggregation of β-CS and CAT. The encapsulation efficacy of CAT or CAT–Zn complex loaded β-CS NPs at the different ratios of β-CS:CAT or CAT–Zn complex was 50–84% and 53–89%, respectively, all decreased along with increased ratios of β-CS:CAT or CAT–Zn complex (Table 2), probably because the low concentration of CAT or CAT–Zn complex led to the higher contact area and degree of crosslinking of β-CS with sodium TPP. The redundant CAT or CAT–Zn complex at high ratios of β-CS:CAT or CAT–Zn complex was retained in the supernatant during centrifugation, resulting in low encapsulation efficacy of CAT or CAT–Zn complex loaded β-CS NPs.
3.3. UV–vis spectra scanning of CAT and CAT–Zn complex and NPs samples

UV–vis spectra scanning of CAT and CAT–Zn complex at the different ratios of β-CS to CAT or CAT–Zn complex are shown in Fig. 2. The new peak appeared in 430 cm$^{-1}$ indicated that Zn has been incorporated into the molecular structure of CAT (Fig. 2B). The absorption peak of CAT changed after CAT and Zn were combined with a new peak appeared at 430 cm$^{-1}$. The prepared CAT or CAT–Zn complex loaded β-CS NPs solutions at the different ratios of CAT or CAT–Zn complex to β-CS are illustrated in Fig. 2C, where the color of solution became a little bit darker with increased amount of CAT or CAT–Zn complex. This might be explained by the aggregation of NPs and the original color of CAT and CAT–Zn complex (Fig. 2C).

Table 2

<table>
<thead>
<tr>
<th>Treatment group$^*$</th>
<th>Particle size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta-potential (mV)</th>
<th>Yield (%)</th>
<th>Encapsulation efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_0$</td>
<td>79.47 ± 2.27$^{++}$</td>
<td>0.329 ± 0.009$^b$</td>
<td>50.36 ± 4.46$^c$</td>
<td>38.76 ± 4.66$^{b}$</td>
<td>–</td>
</tr>
<tr>
<td>$T_1$</td>
<td>115.60 ± 0.85$^a$</td>
<td>0.347 ± 0.146$^b$</td>
<td>44.55 ± 0.63$^{ab}$</td>
<td>57.41 ± 1.31$^{a}$</td>
<td>84.35 ± 7.29$^{d}$</td>
</tr>
<tr>
<td>$T_2$</td>
<td>188.63 ± 1.59$^b$</td>
<td>0.386 ± 0.0017$^b$</td>
<td>43.99 ± 4.38$^{ab}$</td>
<td>35.40 ± 4.14$^a$</td>
<td>70.24 ± 2.31$^b$</td>
</tr>
<tr>
<td>$T_3$</td>
<td>627.03 ± 61.0$^d$</td>
<td>0.421 ± 0.009$^c$</td>
<td>43.67 ± 2.38$^{ab}$</td>
<td>19.63 ± 0.60$^b$</td>
<td>50.32 ± 3.26$^d$</td>
</tr>
<tr>
<td>$T_4$</td>
<td>208.00 ± 16.18$^b$</td>
<td>0.377 ± 0.0093$^b$</td>
<td>39.17 ± 1.60$^{ab}$</td>
<td>55.53 ± 1.71$^e$</td>
<td>89.37 ± 5.38$^d$</td>
</tr>
<tr>
<td>$T_5$</td>
<td>479.30 ± 5.78$^e$</td>
<td>0.387 ± 0.0087$^c$</td>
<td>37.51 ± 2.89$^a$</td>
<td>25.31 ± 1.35$^d$</td>
<td>75.25 ± 7.34$^{ab}$</td>
</tr>
<tr>
<td>$T_6$</td>
<td>590.70 ± 78.70$^d$</td>
<td>0.395 ± 0.00029$^d$</td>
<td>45.63 ± 1.62$^{bc}$</td>
<td>9.11 ± 0.68$^a$</td>
<td>53.25 ± 2.39$^d$</td>
</tr>
</tbody>
</table>

$^*$ $T_0$: control (β-chitosan nanoparticles solutions without catechins or catechins–Zn); $T_1$: β-chitosan:catechins (1:1); $T_2$: β-chitosan:catechins (1:3); $T_3$: β-chitosan:catechins (1:5); $T_4$: β-chitosan:catechins–Zn complex (1:1); $T_5$: β-chitosan:catechins–Zn complex (1:3); $T_6$: β-chitosan:catechins–Zn complex (1:5).
$^{++}$ In the same column, values with the same superscript letter (a–e) were not significantly different (P > 0.05). Data were mean of three replication ± standard deviation.
3.4. Stability of β-CS NPs solution and CAT or CAT–Zn complex loaded β-CS NPs solutions at different particle sizes and different pH

Fig. 3 shows the variations in the turbidity of freshly prepared CAT or CAT–Zn complex loaded β-CS NPs solutions prepared at the different ratios of CAT or CAT–Zn complex to β-CS and different pH. The instability of NPs solution was defined as the increased transmittance (%T) of the solutions (Muzolf-Panek, Gliszczynska-Swiglo, Szymusiak, & Tyrakowska, 2012), in which the increased turbidity of β-CS NPs solution indicates the deprotonation of β-chitosan and/or aggregation of β-CS NPs. In the pH range of 2.0–4.5, all CAT or CAT–Zn complex loaded β-CS NPs solutions were stable because most phosphate groups from sodium TPP were in the form of O\(_{10}P\)\(_{5}\)\(^{-}\) and the amine groups from β-CS were in the form of NH\(_{3}\)\(^{+}\). The results suggested that the NPs were very stable in the pH range of 2.0–4.5. However, when the pH of NPs solution was greater than 5.0, i.e., greater than pKa of β-CS, precipitation in the solutions occurred. Moreover, the aggregation of β-CS NPs would occur at high pH due to the high surface charge. These results were consistent with the previous study (Tang et al., 2013).

3.5. FT-IR analysis

The spectrum of β-CS, sodium TPP, CAT, CAT–Zn complex, and CAT or CAT–Zn complex loaded β-CS NPs are characterized by FT-IR spectroscopy, which were then utilized to examine the possible interaction mechanisms between the sodium TPP, β-CS, CAT and CAT–Zn complex (Fig. 4). The solid β-CS powders had the characteristic peaks at 3,433 (–OH and –NH\(_{2}\) stretching), 2,920 (–CH stretching), 1,640 (amide I) and 1,095 (–C–O–C stretching) (Fig. 4A). For β-CS NPs, the peak of amide I (–NH\(_{2}\) bending) shifted from 3,433 to 3,429 cm\(^{-1}\), and the band became less wide, indicating reduced hydrogen bonding (Fig. 4A(a) and B(d)). CAT had the characteristic peaks at 3,355 (–OH and –NH\(_{2}\) stretching), 1,628, 1,521, 1,467 (ring stretching vibration), and 1,287 cm\(^{-1}\) (–C–O–C stretching) (Fig. 4B(c) and (d))). The band at 1,147 cm\(^{-1}\) presented the stretching vibration of –C–H in benzene ring. However, the band of CAT–Zn complex at 1,628 and 3,355 cm\(^{-1}\) was weakened compared with that of CAT (Fig. 4B(d)), which might be caused by the –C–O stretching in CAT when combined with Zn ion.

The reduced amount of hydrogen bonding in the cross-linked NPs complexes (Fig. 4C) was probably due to more open structure resulting from the crosslinking with sodium TPP (Rodrigues, Costa, & Grenha, 2012). The new peaks appeared at 1,558 cm\(^{-1}\) (amide II), and the band at 808 cm\(^{-1}\) in the NPs might be attributed to the shift
in the 897 cm⁻¹ band of sodium TPP, indicated the complex formation via electrostatic interaction between NH₃⁺ group of CS and phosphoric group of sodium TPP within the NPs (Ji et al., 2011). For CAT loaded β-CS NPs, the peak at —OH and —NH₂ stretching became weak compared with control group. Moreover, the intensity of all characteristic peaks appeared in CAT loaded β-CS NPs became weak along with increased ratios of β-CS to CAT, showing that CAT had been incorporated into β-CS NPs (Fig. 4C((f), (g) and (h))). The band at 1,080 cm⁻¹ exhibited the characteristic absorption peak of —C—O—C stretching appeared in CAT–Zn complex loaded β-CS NPs, indicating that CAT–Zn complex was also incorporated into β-CS NPs (Fig. 4D((i), (j) and (k))).

3.6. Antibacterial activity of β-CS NPs and CAT or CAT–Zn complex loaded β-CS NPs

3.6.1. Growth curve of bacterial strains

The growth curves of L. innocua in sterile Bacto™ Brain Heart infusion and E. coli in Tryptic soy broth containing β-CS NPs or CAT or CAT–Zn complex loaded β-CS NPs at different particle sizes are exhibited in Fig. 5. In general, CAT–Zn complex loaded β-CS NPs showed stronger antibacterial activity than that of CAT loaded β-CS NPs under the same conditions, except for the antibacterial activity against L. innocua at the β-CS NPs to CAT–Zn ratio of 1:5 (T₅₀). The antibacterial activity of CAT or CAT–Zn complex loaded β-CS NPs was higher than that of CAT or CAT–Zn complex alone, especially at the β-CS to CAT or CAT–Zn ratios of 1:1 and 1:3. However, it was noticed that for L. innocua, β-CS showed higher inhibition than all treatment groups except the CAT–Zn complex loaded β-CS NPs at β-CS to CAT–Zn ratio of 1:1 (T₅₀, where the smallest particle size obtained) (Fig. 5B). This result might be explained as the positively charged β-CS interacted more effectively with the negative residual charge on the surface of bacterial cells at the concentration of 1 mg/mL. The β-CS NPs (T₅₀) also showed better antibacterial activity against L. innocua than most of CAT or CAT–Zn complex loaded β-CS NPs. This might be because β-CS NPs had smaller particle size (79.47 nm) than other samples, thus higher affinity with bacteria cells for a quantum–size effect (Ajitha, Ashok Kumar Reddy, & Sreedhara Reddy, 2015). The observed greater antibacterial activity of NPs might be explained by the larger surface area of the NPs, which could be tightly adsorbed onto the surface of the bacteria cells to disrupt the membranes, leading to the leakage of intracellular components for inactivating the bacteria cells (Moritz & Geszke-Moritz, 2013). Moreover, the higher antibacterial activity observed on CAT–Zn complex loaded β-CS NPs of the smallest particle size (T₅₀) might be attributed to the higher encapsulation efficacy of CAT–Zn complex. To quantify the antibacterial activity of CAT or CAT–Zn complex loaded β-CS NPs, MIC and MBC of the samples were further analyzed and reported in the following section.

3.6.2. MIC and MBC of β-CS NPs and CAT or CAT–Zn complex loaded β-CS NPs

The MIC and MBC of β-CS, CAT, CAT–Zn complex, β-CS NPs, and CAT or CAT–Zn complex loaded β-CS NPs against E. coli and L. innocua are reported in Table 3. The MIC and MBC of the smallest particle size of CAT–Zn complex loaded β-CS NPs (T₅₀) was 0.063 and 0.125 mg/mL for E. coli and L. innocua, respectively, lower than that of the large particle size of CAT or CAT–Zn complex...
Table 3
Inhibition zone, MIC, and MBC of β-chitosan, catechins, catechins–Zn complex, catechins and catechins–Zn complex loaded β-chitosan nanoparticles against E. coli and L. innocua.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Inhibition zone (mm)</th>
<th>MIC** (mg/mL)</th>
<th>MBC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>L. innocua</td>
<td>E. coli</td>
</tr>
<tr>
<td>β-Chitosan solution</td>
<td>11.34 ± 0.71abc</td>
<td>9.63 ± 0.16abc</td>
<td>0.125</td>
</tr>
<tr>
<td>Catechins solution</td>
<td>9.65 ± 0.28ab</td>
<td>8.25 ± 0.32a</td>
<td>0.250</td>
</tr>
<tr>
<td>Catechins–Zn complex solution</td>
<td>9.71 ± 0.38ab</td>
<td>11.75 ± 1.26b</td>
<td>0.250</td>
</tr>
<tr>
<td>T0</td>
<td>8.96 ± 0.67a</td>
<td>8.90 ± 0.85b</td>
<td>0.125</td>
</tr>
<tr>
<td>T1</td>
<td>9.50 ± 0.67ab</td>
<td>10.42 ± 0.52bc</td>
<td>0.125</td>
</tr>
<tr>
<td>T2</td>
<td>9.99 ± 0.80abc</td>
<td>10.27 ± 0.59gh</td>
<td>0.125</td>
</tr>
<tr>
<td>T3</td>
<td>9.98 ± 0.05abc</td>
<td>9.47 ± 0.37ef</td>
<td>0.125</td>
</tr>
<tr>
<td>T4</td>
<td>11.69 ± 1.41abc</td>
<td>10.33 ± 1.19gh</td>
<td>0.063</td>
</tr>
<tr>
<td>T5</td>
<td>10.70 ± 1.41c</td>
<td>8.96 ± 0.42gh</td>
<td>0.125</td>
</tr>
<tr>
<td>T6</td>
<td>9.94 ± 0.12abc</td>
<td>9.40 ± 1.02gh</td>
<td>0.125</td>
</tr>
</tbody>
</table>

* T0: control (β-chitosan nanoparticles solutions without catechins or catechins–Zn), T1: β-chitosan:catechins (1:1); T2: β-chitosan:catechins (1:3); T3: β-chitosan:catechins (1:5); T4: β-chitosan:catechins–Zn complex (1:1); T5: β-chitosan:catechins–Zn complex (1:3); T6: β-chitosan:catechins–Zn complex (1:5).
** In the same column, values with the same superscript letter (a–c) were not significantly different (P > 0.05). Data were mean of three replications ± standard deviation. All samples were smashed and the concentration of all samples was 1 mg/mL.

The MIC and MBC values of all samples against L. innocua were lower than that against E. coli, indicating stronger antibacterial activity against L. innocua. These results could be explained by the fact that the outer membrane of Gram-positive bacteria consists of lipopolysaccharides containing phosphate and pyrophosphate groups, rendering the cell surface negatively charged (Moritz & Geszke-Moritz, 2013), so that it could be attached to a cationic chain of CS polymer through more effective electrostatic interaction than Gram-negative bacteria did. The MIC of CAT or CAT–Zn complex loaded β-CS NPs was lower than that of CAT or CAT–Zn complex alone. The MIC of CAT loaded β-CS NPs against E. coli and L. innocua were 0.125 and 0.063, respectively, while the MIC of CAT–Zn complex loaded β-CS NPs against E. coli and L. innocua were 0.063–0.125 and 0.031, respectively, where the small particle size of sample (T4) showed the highest antibacterial activity. Several mechanisms might explain the high antibacterial activity of CAT–Zn complex loaded β-CS NPs of small particle size (Fig. 6). One was that the CAT–Zn complex loaded β-CS NPs of small particle size can lead to an increase in the specific surface of a bacterial specimen, inducing an increase in their ability to enter into the inside of the stains and interrupt the synthesis of strains protein, thus improving antibacterial activity (Nakayama et al., 2012). Second, CAT–Zn complex had higher distribution of electron cloud and bioactivity than CAT (Zhang & Zhao, 2015), and can interrupt/destroy the lipid bilayer membrane of bacteria, resulting in the leakage of intracellular content. Another possible mechanism is that CAT–Zn complex loaded β-CS NPs of small particle size can inhibit cell metabolism, thus leading into respiratory inhibition of bacteria. Furthermore, the small particle size of NPs could play key role on the uptake and secretion of substrates for inhibiting enzyme activity and killing the strains. However, other possible mechanisms related to the bactericidal action of CAT or CAT–Zn complex still need to be further studied.

3.6.3. Inhibition zone
The inhibition zone of β-CS against E. coli and L. innocua was 11.34 and 9.63 mm, respectively, while that of CAT–Zn complex against E. coli and L. innocua was 9.71 and 11.75 mm, respectively, higher than that of CAT (9.65 and 8.25, respectively) (Table 3). In general, samples showed higher inhibition on E. coli than on L. innocua. However, the MIC and MBC results of β-CS showed better antibacterial activity against L. innocua than against E. coli. This difference might be explained by the experimental procedures applied in the inhibition zone test, where β-CS solution was added onto the filter papers placed on the Agar culture medium for the diffusion of solution, which could cause experimental errors. Therefore,
MIC and MBC measurements might be more reliable than that of inhibition zone test. The inhibition zone of CAT–Zn complex loaded β-CS NPs prepared at β-CS to CAT–Zn complex ratios of 1:1, 1:3 and 1:5 was 11.69, 10.70 and 9.94 mm, respectively (Table 3). Note that β-CS concentration used in the inhibition zone test was 1 mg/mL, while 0.25 mg/mL of β-CS was used in the preparation of CAT or CAT–Zn complex loaded β-CS NPs. This different β-CS concentrations might help explain not significantly higher inhibition zone of CAT or CAT–Zn complex loaded β-CS NPs than that of β-CS solution. The CAT–Zn complex loaded β-CS NPs exhibited higher inhibition zone than that of CAT loaded β-CS NPs at the β-CS:CAT or CAT–Zn complex ratios of 1:1 and 1:3 against E. coli. Basically, CAT–Zn complex loaded β-CS NPs of the smallest particle size showed higher inhibition zone (11.69 nm on E. coli) than that the CAT–Zn complex loaded β-CS NPs of large particle size (9.5 nm on E. coli) (Tables 1 and 3). However, there was no difference in the inhibition zone between CAT or CAT–Zn complex loaded β-CS NPs against L. innocua (P > 0.05). The inhibition zone results did not show clear trend on the antibacterial activity against E. coli and L. innocua, while the results of MIC and MBC showed higher antibacterial activity against L. innocua than E. coli.

4. Conclusions

The CAT or CAT–Zn complex loaded β-CS NPs at different particle sizes were prepared and showed good stability at a low pH environment of 2.0–4.5. The antibacterial activity of CAT–Zn complex loaded β-CS NPs against E. coli and L. innocua was higher than that of CAT loaded β-CS NPs. Also, the CAT or CAT–Zn complex loaded β-CS NPs of small particle size exhibited higher antibacterial activity than that of larger particle size of samples because the small size of particles could more easily permeate into the cells resulting in the leakage of intracellular substances and interrupting the synthesis of cell membranes and intracellular protein, thus leading to the death of bacteria strains. For further understanding of the inhibition mechanisms of the encapsulators against enzyme activity and microbial survival and growth, more studies are necessary with the help of advanced analytical methods and apparatus. Moreover, the microscopic changes occurred in the intercellular matrix of strains based on the antibacterial test should be studied in more depth. Further studies should also be emphasized on extending the storage period of CS nanoparticles due to their easy precipitation during storage at room conditions. This study demonstrated that CAT–Zn complex loaded β-CS NPs can potentially be a good candidate of antibacterial carrier for food and biological applications, either direct addition into food or incorporated in food packaging materials.

Acknowledgements

This work was financially supported by the Key Discipline Talented Special Foundation of Shanghai Jiao Tong University (GKWS2107215001), Agri-X project (AF1500028/006) and China Scholarship Council (201406230202).

References


Food characterization 


