The Mekong Delta, Vietnam has traditionally been the major region of aqua produce accounting for over 52% of national aqua crop, and 65% of aqua export. In 2018, the aqua export value was over US$ 8.8 billion dollars, to which export of shrimp contributed 45%. However, pathogen infections directly influence the production and value-added contribution of shrimp in the region. The most common and serious infection is Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome – AHPNS caused by Vibrio parahaemolyticus. It has caused a severe economic loss for shrimp industry in various countries such as Vietnam (Oanh et al., 2013), Malaysia (Kua et al., 2016), Thailand (Joshi et al., 2014), etc.

Copper nanoparticles (CuNPs) have attracted attention of researchers in recent years owing to their low-cost, abundance and remarkable antimicrobial activity. Previous studies have reported antimicrobial properties of CuNPs against Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Aspergillus flavus, Aspergillus niger, Candida albicans (Ramyadevi et al., 2012; Khalaji et al., 2017), Corticium salmonicolor (Cao et al., 2014), Fusarium Oxysporum, Phytophthora Capsici (Pham et al., 2019).

Although CuNPs have been reported to have effective antimicrobial activity, the instability of copper in aqueous solution can be a limitation (Valodkar et al., 2011). CuNPs are either unstable, or prone to surface oxidation that can significantly affect their optical properties. As a result of surface oxidation, the plasmonic properties of CuNPs have not received much attention compared to silver plasmonic properties (Tiwari et al., 2013; Mori et al., 2018). The stabilizers of colloid play an important role in controlling their dispersion stability for application (Huang et al., 2004). Therefore, the synthesis of copper nanoparticles in a plausible polymer matrix has received much attention. Among the wide variety of polymer matrices, biopolymers are often the first choice as naturally available, cheaper as well as easy to synthesize and modify for various applications and more
N.-Y. NGUYEN ET AL.

imporantly, they are environmentally friendly. Chitosan, a transformed polysaccharide obtained by deacetylation of natural chitin, is known as an antibacterial agent, insecticidal agent, biocompatible and biodegradable polymer. Development of copper nanoparticles/chitosan composite (CuCS) have been attractive because of their perfect antibacterial activity and the unique properties of chitosan (Li et al., 2013; Tabesh et al., 2018; Prokhorov et al., 2019).

There are just few studies about inactivation of V. parahaemolyticus using metal nanoparticles. Ravikumar et al. (2012) firstly investigated the effect of five commercial nanoparticles such as Al₂O₃, Fe₃O₄, CeO₂, ZrO₂, and MgO on V. parahaemolyticus. Among the five nanoparticles, only MgO nanoparticles showed antibacterial activity against V. parahaemolyticus. The effect of silver nanoparticles against V. parahaemolyticus was also investigated (Kandasamy et al., 2013; Zarei et al., 2014). To the best of our knowledge, there is no report on inactivation of V. parahaemolyticus using copper nanoparticles/chitosan composite (CuCS). To achieve a stability of CuNPs in antibacterial applications, in this study, a simple green approach synthesis was developed. CuNPs were synthesized using L-ascorbic acid as a green reducing agent and chitosan as a biopolymer matrix and stabilizing agent. The physical properties of CuCS were evaluated. Then, antibacterial activity of CuCS against V. parahaemolyticus was conducted.

Copper (II) chloride (CuCl₂, 99.0%), acetic acid (C₂H₄O₂, 99.5%), L(+) Ascorbic acid, (C₆H₈O₆, 99%) and sodium hydroxyl (NaOH, 98%) were purchased from Across Organic. Chitosan (85% deacetylated, low molecular weight) was purchased from Himedia (India), and deionized (DI) water was used throughout the experiment. All chemicals were used without further purification.

The CuCS was prepared from chitosan and copper chloride. The typical preparation was as follows: 20 mL of 0.05 M CuCl₂ solution before mixing with 50 ml chitosan solution was purge with nitrogen gas for 60 min. The mixture was stirred at ambient temperature for another 60 min and then adjusted to pH 6.5 by using 0.1 M NaOH solution. The mixture was then refluxed at 80 °C for 60 min with stirring and purging nitrogen gas. Another mixture containing 50 mL ascorbic acid solution (0.02M) was also prepared by purging nitrogen gas for 60 min and adjusting to pH 6.5. The prepared ascorbic acid solution was purged with nitrogen gas for 60 min and adjusted to pH6.5 before introducing drop wise to the CuCl₂. The obtained mixture was left stirring at 80 °C for 4 h for complete reduction to obtain the viscous liquid with brick red light color which is named as CuCS. Finally, the CuCS was allowed to cool to room temperature and purging nitrogen gas was stopped.

Detail of prepared chitosan solutions with different concentrations were prepared as follows. A weighted 0.25, 0.50, 0.75, 1.00 or 1.25 g chitosan was dissolved in 200 mL acetic acid solution (1 wt%) and stirred at 50 °C for overnight to form solution with various chitosan concentrations. A separated 50 mL of chitosan solution was introduced to a 20 mL CuCl₂ solution, as mentioned above, to get the final CuCS sample with different ratio of chitosan over CuCl₂ solution (w/v) of 0.25, 0.50, 0.75, 1.00 and 1.25.

The crystallinity and phase composition of the synthesized CuCS were investigated by X-Ray D8 Advance X-ray diffractometer, using CuKα radiation with the wavelength λ =1.5418 Å at scanning steps of 0.02° in the scanning range of 20° to 80°. UV-Vis absorption spectra of CuCS were recorded from 450 to 750 nm using an ultraviolet-visible absorption spectroscopy (Horiba-Dual Fl). The morphology of obtained CuCS was observed by a transmission electron microscope (TEM, TEM Jeol 1010b). From the TEM images, the distribution of particle size was calculated using the software of ImageJ and tool bar of ROI Manager. The optical density at 600 nm (OD₆₀₀) was recorded by UV-Vis spectrophotometer (CT-2200, ChromTech, Taiwan).

Antibacterial activity test of CuCS against a laboratory V. parahaemolyticus strain was conducted. V. parahaemolyticus TS2 was obtained from Vietnam Institute of Aquaculture II. It was cultivated at 30 °C, 120 rpm in TSB supplemented with of 2% (w/v) NaCl until an OD₆₀₀ of 0.1 (~10⁷ CFU/mL) was attained. The culture was centrifuged at 10,000 x g, 4 °C, 5 min to obtain a pellet. The pellet was resuspended in the same volume of sterilized shrimp-pond water. The centrifugation and suspension were repeated to remove residuals of TSB. The final pellet was resuspended and serially diluted in sterilized shrimp-pond water to obtain a bacterial concentration of approximately 10⁸ CFU/mL. The solution was divided into six 5-mL aliquots in 50-ml tubes. Three aliquots were mixed with 25, 50, or 100 µL of chitosan-copper nanoparticles to obtain a final concentration of 2.5, 5.0, or 10.0 ppm. One aliquot was left blank without chitosan-copper nanoparticles addition (0 ppm). The other two aliquots were mixed with 50 µL ascorbic acid/ chitosan (AA/CS) or CuCl₂. The mixtures were shaken at 30 °C, 120 rpm. Sampling was performed at 0, 2, and 4 h. The sample was serially diluted. Each 100-µL portion was spread onto thiosulfate citrate bile salts sucrose (TCBS) agar, incubated overnight at 30 °C to estimate bacterial concentration. The experiment was conducted in triplicate.

Antibacterial activity test of CuCS in an EMS shrimp-pond water was conducted. A sample was obtained
from a shrimp-feeding pond in Tien Giang province, Mekong Delta, Vietnam on March 14, 2019. Shrimps in the pond at the time of sampling were challenged to EMS infection. The sample was transferred to the laboratory under cold conditions. It was serially diluted and spread onto TCBS agar to confirm existence of V. parahaemolyticus. Next, the solution was divided into two 5-mL aliquots in 50-ml tubes. One aliquot was mixed with 50 µL of chitosan-copper nanoparticles to obtain a final concentration of 5.0 ppm. The other aliquot left blank without CuCS addition (0 ppm). The mixtures were shaken at 30 °C, 120 rpm. Sampling was performed at 0, 2, and 4 h. The sample was serially diluted. Each 100-μL portion was spread onto TCBS agar, incubated overnight at 30 °C to estimate bacterial concentration. The experiment was conducted in triplicate.

Figure 1 shows the effect of chitosan rate, which is presented in ratio of chitosan over CuCl2 solution (w/v) being 0.50, 0.75, 1.00 and 1.25 on the CuNPs formation. The maximum absorption positions of samples with 0.50, 0.75, 1.00 and 1.25 (w/v) were measured to be 593, 584, 582 and 586 nm, respectively. The obtained spectra exhibit an absorption band at approximately 593 to 582 nm, which is typical of plasmon band of copper nanoparticles. Moreover, increasing chitosan rate from 0.50 to 1.0 % w/v resulted in a red shift in the maximum absorption position from 593 (0.50 % w/v) to 584, 582 nm (1.0 % w/v). The blue shift in SPR peak position when increasing chitosan rate could be attributed to the decrease in particle size. The higher concentration of chitosan results a smaller particle size of copper. This could be attributed to the possibility of chitosan covering the particle surface of CuNPs and prevent their growth. However, when further increasing chitosan rate up to 1.25 %, the SPR peak position shift to 586 nm. This increasing due to the high concentration of chitosan may lead to be aggregated of CuNPs. It can be summarized that the smallest size of CuNPs could be synthesized under such conditions as ratio of chitosan over CuCl2 solution (w/v) being 1.00 w/v, molar ratio of ascorbic acid: CuCl2 to be 1:1, pH 7, reduction temperature of 80 °C for 4 h.

The crystal structure and phase composition of the prepared sample were analysed using XRD. Figure 2 shows the XRD patterns of the sample. The XRD peaks appearing at 2θ = 43.2° (111), 50.4° (200), and 74.1° (220) are well indexed to the JCPDS card No. 04-0836 representing the face-centred cubic structure of copper, and this confirmed the obtained single phase of CuNPs.

The morphology of synthesized CuCS was carried out by TEM image as depicted in Figure 3. The obtained CuCS show wide distribution of particle size and aggregation. Figure 4 represents the FT-IR spectrum of CuCS. The spectrum shows its characteristic bands at 3425 cm⁻¹ and 2920 cm⁻¹ which can be attributed to O-H and N-H stretching, respectively. The bands at 1643 cm⁻¹ and 1411 cm⁻¹ are related to N-H and C-O-H bending, respectively. A broadband at 1068 cm⁻¹ with a shoulder at 1157 cm⁻¹ is assigned to C-O stretches and C-O-C of saccharide ring, respectively (El-Aziz et
One broadband emerged at 3293 cm$^{-1}$ in the spectrum of CuCS, which appeared as two separate bands in CS spectrum. Finally, it can be concluded that the CuCS are successfully synthesized at ratio of chitosan over CuCl$_2$ solution (w/v) being 1.00 w/v, molar ratio of ascorbic acid: CuCl$_2$ to be 1:1, pH 7, reduction temperature of 80 °C for 4 h. This obtained CuCS are used for the further evaluation of antibacterial activity.

For testing antibacterial activity of CuCS against the laboratory $V. \text{parahaemolyticus}$ strain, a suspension of $V. \text{parahaemolyticus}$ cells in sterilized shrimp-pond water was prepared at an initial concentration of approximately $10^5$ CFU/mL. As described in Figure 5, in the solution containing CuCl$_2$ or ascorbic acid/chitosan (AA/CS), no antimicrobial activity was shown after 2 h and 4 h. Concentration of bacterial cells slightly increased in these cases because of remaining nutrients in shrimp-pond water. It indicated that the materials used to synthesize CuCS did not inhibit growth of bacterial cells. In contrast, following challenge with CuCS, the bacterial count sharply decreased over 4 h of incubation. Elimination percentage at 2.5 ppm was 91.47% and 95.26% after 2 and 4 h, respectively. Elimination percentage at 5.0 ppm was 100% after 2 h when there were no colonies formed on TCBS agar plates spread by 100 μL of sample without dilution. The detection limit of agar-plate method was considered as 10 CFU/mL. It indicated minimum bactericidal concentration (MBC) of CuCS was 5.0 ppm (Wei et al., 2009).

For examining antibacterial activity of CuCS against $V. \text{parahaemolyticus}$ in the EMS real shrimp-pond water, the existence of $V. \text{parahaemolyticus}$ in the shrimp-feeding pond was shown on TCBS agar plates (green colonies) (Figure 6; 0h). The initial concentration of $V. \text{parahaemolyticus}$ in the sample was approximately $2 \times 10^2$ CFU/mL. CuCS was applied to the solution at 5.0 ppm. After 2-h incubation, no colonies was formed on the plates spread by 100 μL of sample without dilution. In contrast, bacterial count in the control without CuCS addition remained constant. It indicated that a high efficacy of CuCS in elimination of $V. \text{parahaemolyticus}$ in a real shrimp-feeding pond.

Some studies have showed high resistance rates of 90% – 100% of aquatic bacterial pathogens to common antibiotics (Nguyen et al., 2016). This has had alarming ramifications including output reduction and loss of export potential. Usage of metal nanoparticles is considered as alternative to antibiotics in aquaculture due to their high surface area, less toxicity, and heat resistance (Brayner et al., 2006). Copper nanoparticles are considered as safer to humans and animals than other metal nanoparticles because excessive amounts of copper in human and animal cells can be exported by copper-transporting adenosine triphosphatases (Cu-ATPases) (Lutsenko et al., 2007). In addition, copper is cheaper than other noble metals such as silver and gold. Therefore, studies on the usage of copper nanoparticles against human pathogenic bacteria have been largely conducted (Ramyadevi et al., 2012; Cao...
et al., 2014; Singh et al., 2016; Khalaji et al., 2017). However, studies investigating antibacterial activity of copper nanoparticles against shrimp pathogenic bacteria especially *V. parahaemolyticus* are very limited. There are just a few reports about inactivation of *V. parahaemolyticus* using metal nanoparticles other than CuNPs (Ravikumar et al., 2012; Kandasamy et al., 2013; Zarei et al., 2014). The current study is considered as the...
first report on inactivation of *V. parahaemolyticus* using copper nanoparticles.

Antibacterial property of metal nanoparticles against Gram-positive bacteria is demonstrated to be higher compared to Gram-negative bacteria (Premananthan et al., 2011; Ravikumar et al., 2012; Azam et al., 2012; Zain et al., 2014). This might be because the cell-wall structure of Gram-negative bacteria is more complex than that of Gram-positive bacteria (Zain et al., 2014). *V. parahaemolyticus* is expected not to be easily treated since it is a Gram-negative bacterium. However, CuCS synthesized in the current study has shown a high antibacterial activity against *V. parahaemolyticus*. It completely eliminates *V. parahaemolyticus* at 5.0 ppm after 2 h of exposure in the real EMS-infected shrimppond water. These findings suggest that CuCS can be competitive to other metal nanoparticles. Silver nanoparticles have been reported to show a MBC of 6.26 ppm to different four pathogens including *V. parahaemolyticus* (Zarei et al., 2014), CeO$_2$ nanoparticles showed a MIC of 20 and 30 ppm against *Bacillus subtilis* and *V. harveyi*, respectively (Ravikumar et al., 2012). Stability of copper nanoparticles by chitosan contributes to the high efficiency of CuCS against *V. parahaemolyticus* in the current study as indicated previously (Valodkar et al., 2011). It implies that the CuCS might be used as an alternative antibacterial agents for the EMS control in shrimp farming in the Mekong delta, Vietnam. This assumption will be examined in the future studies.

In conclusion, *V. parahaemolyticus* has recently caused a serious output reduction and export loss for shrimp industry in the Mekong delta, Vietnam, due to the high antibiotic resistance rate of this pathogen. The ultimate goal of the study is to investigate the capacity of CuCS as an alternative solution to antibiotics for treating the EMS infection. A high antibacterial activity of CuCS against *V. parahaemolyticus* in vitro was initially reported at as low as 2.5 and 5.0 ppm. In addition, the use of L-ascorbic acid as a green reducing agent and chitosan as a biopolymer matrix will result in environmentally friendly materials. Therefore, CuCS is expected to be highly applicable for treating the EMS infections in shrimp farms in the region.

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