Influence of salt addition and freezing-thawing on particle size and zeta potential of nano-chitosan

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Abstract. Antibacterial properties of nano-chitosan used for fish preservation would achieve optimum effect when combined with cooling. Applying nano-chitosan incorporated in ice can reduce the cooling cost of conventional fish industry. On the other hand, during fish handling, nano-chitosan has a high probability to be contaminated by salt in seawater. This study was aimed to test the effect of salt and freezing-thawing on nano-chitosan antibacterial activity. Nano-chitosan was prepared using ionic gelation and polyelectrolyte complex methods. NaCl (3% m/v) was added into nano-chitosan solutions to evaluate the effect of salt. Chitosan solution in diluted acetic acid was also tested as blank. The effect of freezing was undertaken by placing nano-chitosan and chitosan solution in the freezer until the solutions were completely frozen and then thawing the frozen solution in ambient temperature. The addition of salt not only reduced the particle size but also reduced zeta potential due to the possible neutralization. It also might reduce its antibacterial activity. Freezing effect increased particle size because low temperature triggered particle agglomeration but it did not show any changes in zeta potential. The result of this preliminary study indicated that nano-chitosan ice would give more benefit as fish preservative compared to plain ice.

Keywords: chitosan, freezing, salt

1. Introduction

Chitosan is a natural material derived from chitin by deacetylation process. As a pseudo-natural cationic polymer, chitosan has unique characteristics due to the activity of NH3+ on its structure that appears when chitosan is dissolved in an acidic solution [1]. The active group of chitosan is able to interact with the bacterial cell membrane so that it inhibits bacterial growth [2–5]. The antibacterial property of chitosan gives beneficial effects for preserving food. Chitosan is proven to inhibit the deterioration of fish quality [6–9]. Several studies denote that the antibacterial properties of chitosan increase by changing the size of the chitosan into nano-particles [10–12]. Nano-chitosan is also evident to delay the degradation of fish quality [11,13,14].

Nano-chitosan is produced by dissolving chitosan into a diluted acetic acid solution and then crosslinker is added to form particles. The ionic gelation method uses tripolyphosphate (TPP) as a crosslinker while the complex polyelectrolyte method uses oligosaccharides [15]. The TPP route
resulted in a significant antibacterial activity [10] and a stable formulation [16] but is still under discussion as a preservation agent in fishery products because it may result in economic fraud [17]. Polyelectrolyte complex becomes an alternative to nano-chitosan formulations since it uses oligosaccharides such as Arabic gum as crosslinker [18]. The complexity of chitosan and Arabic gum also showed a good antibacterial activity [19].

The positive charge of chitosan is more concentrated when converted to a nanoparticle therefore will influence the permeability of bacteria membrane cell and interrupt bacterial growth more [2]. Charges in the colloids show the presence of electro-kinetic potential which is called zeta potential [20]. Beside describing the level of electro-kinetic charge, zeta potential also provides information on the stability of the nano-chitosan in colloid due to the electrostatic repulsion that appears from similar charges, prevents agglomeration. Zeta potential can be affected by the presence of counter ion from other ions added to the solution [21]. Fish handling practices on the conventional fisherman’s ship is a challenge for nano-chitosan application due to the possible contamination by seawater that contains salt. Besides that, one of the types of fish product connects with salt. For example, salted fish uses salt to marinate. Salt affects the characteristics of colloidal solutions because of the contribution of positive and negative ions [22].

Utilization of chitosan or nano-chitosan as a preservative agent exhibits optimum activity when it is combined with cooling [23]. In the current practice, traditional fish handling commonly uses a lot of ice and therefore needs a large space. Nano-chitosan in the form of frozen dispersion in water can be an alternative to optimize the shelf life of the product since it inhibits bacterial activity as the ice melted. A previous study suggested to store chitosan solution under 5°C because a significant chain hydrolysis was not noticed [24]. On the other hand, in storing nano-chitosan at 2°C it has been found that an indication of agglomeration occurred [25]. Freezing probably changes the properties of nano-chitosan and the extent of the change also needs to be observed to find out its potential uses.

2. Materials and Methods

2.1. Materials

The chitosan and sodium tripolyphosphate were purchased from Sigma Aldrich and Merck, respectively. The acetic acid glacial was purchased from Ajax Finechem Pty. Ltd. The Mili-Q water was processed with Milli-Q water purifier from Millipore Corporation. Arabic gum was obtained from a local market. The sodium chloride was purchased from Fluka Analytical.

2.2. Preparation of nano-chitosan by ionic gelation and polyelectrolyte complex methods

Chitosan (0.1% w/v) was dissolved in 1% of diluted acetic acid (v/v) to obtain a chitosan solution. After a homogenization process for 2 hours, the chitosan solution was divided into three parts. One part was added with TPP solution (0.84 g/L), the second one with Arabic gum (3 g/L) and the last one with Mili-Q water (as comparison) by using the volume ratio of 5:2 (chitosan solution:crosslinker) in all variations. Each mixture was homogenized for 30 minutes. All solutions were then kept in room temperature until they were used in experiments.

2.3. Preparation of salt treatment

All three solutions of chitosan (chitosan-TPP, chitosan-arabic gum, chitosan solution) were treated in pairs, i.e. one with the addition of salt and the other without salt. Seawater contains salt approximately 3.5% (g/L) [26] and it is dominated by NaCl. It was used as a basis for salt addition into the solution. The 3% of NaCl was added to all solutions and the resulting mixtures were analyzed for particle size, zeta potential and pH value.

2.4. Preparation of freezing treatment

The three treated solutions were also compared for the particle size, zeta potential and pH value before and after freezing-thawing. Freezing-thawing treatment was conducted by firstly putting solutions in conical tubes with 50 mL volume and frozen in -20°C for 2 days. After freezing, the
solution was defrosted in room temperature (25°C) and the liquid was analyzed for the particle size, zeta potential and pH value.

3. Results and Discussion

3.1. Effect of salt on the of nano-chitosan and chitosan solution characteristics

Nano-chitosan has been applied for preservation of different fishery products, such as Litopenaeus vannamei (whiteleg shrimp) [13], Hypophthalmichthys molitrix (silver carp) fillet [11] and finger fish [14]. All studies showed that nano-chitosan was able to extend the shelf life of those products by inhibiting bacterial growth. This result showed a promising opportunity for nano-chitosan to be applied to other products. One of the types of fish products strongly related with salt. For example, the salted fish use salt to marinate. Another application on fish handling on the sea is also posed to a very high chance of contamination with seawater containing salt. The effect of salt in the stability of particle size of nano-chitosan is shown in figures 1 and 2.

![Figure 1](image1.png)

**Figure 1.** Effect of salt on the particle size of nano-chitosan and chitosan solution, no salt; salt.

Particle size is an important aspect since it affects the surface contact area between nano-chitosan and bacteria. The smaller particle size with higher concentration of positive charge will lead to higher antibacterial activity [10]. Chitosan, when dissolved in diluted acetic acid, is protonated so that the electrostatic repulsion force arises and causes molecular swelling. In this study, the swelling process was characterized by large particle sizes of chitosan. The addition of crosslinkers caused a particle size reduction due to crosslinking between chitosan and crosslinkers [27]. The addition of TPP resulted in smaller nano-chitosan particles than Arabic gum. This was probably due to the molecular size of Arabic gum being larger than TPP.

The presence of salt also caused a reduction in the average of particle size (figure 1). This was also found in the study of [28] which added 0.05 M and 0.15 M of NaCl. The salt’s ions reduced the electrostatic repulsion between the amine group of chitosan so that the swelling effect decreased and the particle size became smaller. Besides, Cl⁻ ion from salt triggered the formation of electrostatic screening effect. Typically the negative ion got close to the positive charge of chitosan and forms a screening/shielding effect so that it suppresses the molecular swelling [29]. The molecular swelling inhibition was observed more clearly from the data of particle size distribution. The presence of crosslinkers and NaCl was shifting particle size distribution to the left side towards a smaller range of particle size (figure 2). Moreover, salt addition narrowed the particle size distribution that indicated a good colloidal stability [21, 30].
The zeta potential of the nano-chitosan and chitosan solution ranged above ±30 mV which indicated a good stability before adding salt. Nano-suspension was solely stabilized by an electrostatic repulsion with a minimum of ±30 mV of zeta potential in order to prevent agglomeration [31]. The TPP is an anionic molecule, so if it is mixed into chitosan with abundant positive charge, the final charge of the solution will decrease. This actually also occurred in the complex chitosan-arabic gum mixture. But, due to the pH value of the solution of complex chitosan-arabic gum being below 3.5 (figure 4), the carboxylic groups of Arabic gum also experienced protonation [32] so that the final charge of the solution only decreased slightly. Such a tendency of the contribution from crosslinker to the net charge was also found in the research of [33].

The salt addition led to a significant decrease in the zeta potential of all solutions. The ionized salt weakened the electrostatic interaction between chitosan and the crosslinker [21]. In addition, there was also the possibility of a charge neutralization mechanism because in the chitosan solution without crosslinkers and the solution with salt addition, the zeta potential also decreased. This prediction is supported by the data of pH value which also decreased after the NaCl addition (figure 4). The initial pH value of the chitosan solution was 2.65. This pH value increased after being added with TPP and Arabic gum since TPP and Arabic gum solutions have initial pH of 9.04 and 5.19, respectively. The presence of salt decreases the pH value of all treatments.
Figure 4. Effect of salt on the pH value of nano-chitosan and chitosan solution, □ no salt; ▢ salt.

The low zeta potential generated a low antibacterial activity as well [10]. The effective antibacterial activity was derived from molecules with high zeta potential and small particle sizes. Thus, for the purpose of preserving food that contains salt, nano-chitosan is not suitable.

3.2. Effect of freezing-thawing on the nano-chitosan and chitosan solution characteristics

Cold chain is the main method in handling and preserving fishery products. The nano-chitosan ice was inspired by how to enhance the effectiveness of nano-chitosan and saving the use of ice. This is a combination of hurdle techniques on food preservation because it is expected that there will be a synergistic effect of the two methods while decreasing cost. The freezing process expanded the particle size of all solutions as expected. When the freezing process runs, some water molecules will form crystals, causing the solution to be more concentrated and the viscosity of the solution to increase. The increase in concentration also occurred in a binary system of sucrose-water, in which during freezing the temperature dropped and increased the solution concentration [34]. Under these conditions, the opportunity for agglomeration was higher. This was evident from all solutions having increased particle size (figure 5). As can be seen from the particle size distribution, there was a shift towards a larger direction and tends to form a bimodal pattern (figure 6). The change in the curve from unimodal to bimodal showed a heterogeneity of particle size.

Figure 5. Effect of freezing on the particle size of nano-chitosan and chitosan solution, □ before freezing, ▢ after freezing.
The interesting observation in the freezing process of nano-chitosan and chitosan solutions was the fact that freezing increased zeta potential which will be good to enhance antibacterial properties (figure 7). Zeta potential increased in the freezing-thawing process because of an increase in viscosity that led to more concentrated particles and increased positive charge. The escalation of zeta potential and particle size also occurred in nano-chitosan with and without hyaluronate or alginate coating [35]. Zeta potential of all treatments still appeared in the range of zeta values for good stability.

Currently, the practice of preserving fish during distribution uses ice arranged in a box to cover the fish. The cold temperature of ice will suppress the growth of bacteria which causes quality deterioration in fish. The synergism of two preservation techniques (nano-chitosan and cooling) is expected to extend the shelf life of fish. At the initial phase, the preservation will be dominated by the role of the low temperature of ice. During storage, the ice will thaw and will release nano-chitosan particles and contribute to extend the shelf life of the product. The pH value showed no significant difference, so it was expected to give a similar effect with a fresh solution when applied to the product. This phenomenon opens the opportunity for further research in the use of nano-chitosan ice for fish preservation.
Figure 8. Effect of freezing on pH value of nano-chitosan and chitosan solution, ■ before freezing, ■ after freezing.

4. Conclusion

The presence of 3% salt in nano-chitosan and chitosan solutions appeared to be able to reduce particle sizes but also reduce zeta potential. The application of nano-chitosan for the preservation of salt containing products may not be viable. On the other hand, using nano-chitosan along with a freezing process, which generates a slight increase in particle size, was accompanied by an increase in zeta potential. The maintenance of zeta potential after freezing provided an opportunity for the frozen dispersed nano-chitosan to be tested as antibacterial agent. In the future, further research is needed on the application of each formula for fish preservation.

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References

[1] Rinaudo M 2006 Chitin and chitosan: Properties and applications Prog. Polym. Sci. 31(7) 603–32
[2] Tsai G J and Su W H 1999 Antibacterial activity of shrimp chitosan against Escherichia coli J. Food Prot. 62(3) 239–43
[3] Chung Y, Su Y, Chen C, Jia G, Wang H, Wu J C G and Lin J 2004 Relationship between antibacterial activity of chitosan and surface characteristics of cell wall Acta Pharmacol. Sin. 25(6) 932-936
[4] Liu N, Chen X G, Park H J, Liu C G, Liu C S, Meng X H and Yu L 2006 Effect of MW and concentration of chitosan on antibacterial activity of Escherichia coli Carbohydr. Polym. 64(1) 60–65
[5] Chang S H, Lin H T V, Wu G J and Tsai G J 2015 pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan Carbohydr. Polym. 134 74–81
[6] Jeon Y J, Kamil J Y V A and Shahidi F 2002 Chitosan as an Edible Invisible Film for Quality Preservation of Herring and Atlantic Cod J. Agric. Food Chem. 50(18) 5167–78
[7] Mohan C O, Ravishankar C N, Lalitha K V, Srinivasa and Gopal T K 2012 Effect of chitosan edible coating on the quality of double filleted Indian oil sardine (Sardinella longiceps) during chilled storage Food Hydrocoll. 26(1) 167–74
[8] Fan W, Sun J, Chen Y, Qiu J, Zhang Y and Chi Y 2009 Effects of chitosan coating on quality and shelf life of silver carp during frozen storage Food Chem. 115(1) 66–70
[9] Zhou R, Liu Y, Xie J and Wang X 2011 Effects of combined treatment of electrolysed water and chitosan on the quality attributes and myofibril degradation in farmed obscure puffer fish (*Takifugu obscurus*) during refrigerated storage *Food Chem.* **129**(4) 1660–6

[10] Qi L, Xu Z, Jiang X, Hu C and Zou X 2004 Preparation and antibacterial activity of chitosan nanoparticles *Carbohydr. Res.* **339**(16) 2693–700

[11] Ramezani Z, Zarei M and Raminnejad N 2015 Comparing the effectiveness of chitosan and nanochitosan coatings on the quality of refrigerated silver carp fillets *Food Control* **51** 43–8

[12] Aliasghari A, Khorasgani M R, Vaezifar S, Rahimi F and Khoroushi M 2016 Comparing the effectiveness of chitosan and nanochitosan coatings on the quality of refrigerated silver carp fillets *Food Control* **51** 43–8

[13] Wang Y, Liu L, Zhou J, Ruan X, Lin J and Fu L 2015 Effect of chitosan nanoparticle coatings on the quality changes of postharvest whiteleg shrimp, *Litopenaeus vannamei*, during storage at 4°C *Food Bioprocess Technol.* **8**(4) 907–15

[14] Abdou E S, Osheba A S and Sorour M A 2012 Effect of chitosan and chitosan-nanoparticles as active coating on microbiological characteristics of fish fingers *Int. J. Appl. Sci. Tech.* **2**(7) 158–169

[15] Grenha A 2012 Chitosan nanoparticles-a survey of preparation methods. *J. Drug Target.* **20**(4) 291–300

[16] Morris G A, Castile J, Smith A, Adams G G and Harding S E 2011 The effect of prolonged storage at different temperatures on the particle size distribution of tripolyphosphate (TPP) – chitosan nanoparticles *Carbohydr. Polym.* **84**(4) 1430–4

[17] Gonçalves A A and Ribeiro J L D 2008 Do phosphates improve the seafood quality? Reality and legislation *Pan-Am J. Aquat. Sci.* **3**(3) 237–47

[18] Appolonia I C, Modupe O G, Ayodeji E T and Michael Ol K 2017 Synthesis and characterization of chitosan/gum arabic nanoparticles for bone regeneration *Am. J. Mater. Sci. Eng.* **5**(1) 28–36

[19] Maqbool M, Ali A and Alderson P 2010 A combination of gum arabic and chitosan can control anthracnose caused by *Colletotrichum musae* and enhance the shelf-life of banana fruit *J. Hortic. Sci. Biotechnol.* **85**(5) 432–6

[20] Honary S and Zahir F 2013 Effect of zeta potential on the properties of nano-drug delivery systems - A review (Part 1) *Trop. J. Pharm. Res.* **12**(2) 255-264

[21] Huang Y and Lapsitsky Y 2011 Monovalent salt enhances colloidal stability during the formation of chitosan/tripolyphosphate microgels *Langmuir* **27**(17) 10392–9

[22] López-León T, Carvalho E L S, Seijo B, Ortega-Vinuesa J L and Bastos-González D 2005 Physicochemical characterization of chitosan/gum arabic nanoparticles: electrokinetic and stability behavior *J. Colloid Interface Sci.* **283**(2) 344–51

[23] Budhijanto B, Nugraheni P S and Budhijanto W 2015 Inhibition of microbial growth by nanochitosan for fresh tilapia (*Oreochromis* sp) preservation *Procedia Chem.* **16** 663–72

[24] Nguyen T T B, Hein S, Ng C H and Stevens W F 2008 Molecular stability of chitosan in acid solutions stored at various conditions *J. Appl. Polym. Sci.* **107**(4) 2588–93

[25] Handani W R, Sediawan W B, Tawfïqurrahman A, Wiratni and Kusumastuti Y 2016 The effect of temperature and chitosan concentration during storage on the growth of chitosan nanoparticle produced by ionic gelation method *AIP Conference Proceedings* **1840**

[26] Millero F J, Feistel R, Wright D G and McDougall T J 2008 The composition of Standard seawater and the definition of the reference-composition salinity scale *Deep Sea Res. Part Oceanogr. Res. Pap.* **55**(1) 50–72

[27] Chattopadhyay D P and Inamdar M S 2012 Studies on synthesis, characterization and viscosity behaviour of nano chitosan *Res. J. Engineering Sci.* **1**(4) 9-15

[28] Jonassen H, Kjønniksen A L and Hiorth M 2012 Stability of chitosan nanoparticles cross-linked with tripolyphosphate *Biomacromolecules* **13**(11) 3747–56

[29] Zhang Q, Wu Q, Lin D and Yao S 2013 Effect and mechanism of sodium chloride on the formation of chitosan–cellulose sulfate–tripolyphosphate crosslinked beads *Soft Matter* **9**(43) 10354
[30] Primaningtyas A, Budhijanto W, Fahrurrozi M and Kusumastuti Y 2017 The effects of surfactant and electrolyte concentrations on the size of nanochitosan during storage AIP Conference Proceedings \textbf{1840}

[31] Muller R H, Jacobs C and Kayser O 2001 Nanosuspensions as particulate drug formulations in therapy Rationale for development and what we can expect for the future \textit{Adv. Drug. Deliv. Rev.} \textbf{47} 3–19

[32] Espinosa-Andrews H, Báez-González J G, Cruz-Sosa F and Vernon-Carter E J 2007 Gum Arabic–Chitosan Complex Coacervation \textit{Biomacromolecules} \textbf{8}(4) 1313–8

[33] Tan C, Xie J, Zhang X, Cai J and Xia S 2016 Polysaccharide-based nanoparticles by chitosan and gum arabic polyelectrolyte complexation as carriers for curcumin \textit{Food Hydrocoll.} \textbf{57} 236–45

[34] Abdelwahed W, Degobert G, Stainmesse S and Fessi H 2006 Freeze-drying of nanoparticles: Formulation, process and storage considerations \textit{Adv. Drug. Deliv. Rev.} \textbf{58}(15) 1688–713

[35] Almalik A, Alradwan I, Kalam M A and Alshamsan A 2017 Effect of cryoprotection on particle size stability and preservation of chitosan nanoparticles with and without hyaluronate or alginate coating \textit{Saudi Pharm. J.} \textbf{25}(6) 861–7