Biocoagulant of blood based on chitosan nanoparticle from crustacea

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Abstract. Chitosan is a chitin derivative natural polymer compound isolated from aquaculture waste, such as a crab shell that is the most chitosan source. Nanocitosan as a drug delivery offers delivery with non-harmful routes such as oral, nose, eye, and absorption. Nanokitosan can be absorbed by human organs that have nano-sized membrane penetration capabilities such as the kidneys, liver, and lungs where nanoparticles are 1-1,000 nm in size. Chitosan nanoparticles are prepared by ionic gelation techniques. The mechanism of this method is the formation of chitosan nanoparticles based on the electrostatic interaction between the positive amine groups on chitosan (–NH₂) with the negative charge group of polyanion tripolyphosphinate (TPP). The first step is the preparation of chitosan, crab shell waste which is smoothed to 60 mesh, after subsequent smrubination to be smoothed to remove the protein content by dissolved in 3 N NaOH, then crude chitin demineralized to eliminate the mineral content contained in the crab shell with the addition of HCl 1 N and the presence of heating and stirring. The chitin obtained then enters the deacetylation stage to remove the acetyl group present in the crab shell by adding 50% NaOH and carried out by heating and stirring. The chitosan obtained was then dissolved in 3% lactic acid to obtain liquid chitosan, then added NaTPP to reduce the chitosan particle size to nano size. The obtained nitrocitosan size is 688 nm distribution with variation of NaTPP addition of 0.1% concentration and stirring 60 minutes. The resulting nitrocitosan is tested to increase the rate of blood clotting that has been shown to shorten blood clotting time by up to 30 seconds.

1. Introduction

Based on the results of Household Health Survey (SKRT) conducted by the Ministry of Health mentioned that mouth and dental disease ranked sixth in the suffering community as much as 60 percent of the 10 most diseases in society [1]. People with dental and oral problems who received treatment or treatment from dental health workers were 29.06% and 21.05%. In the age group 10 - 24 years of dental and mouth problems are common. According to the chairman of PGDI, drg. Farichah Hanum, M. Kes said that mouth can be a reflection of signs of systematic disease in the body. Many body disorders whose first signs appear from within the oral cavity. In addition, risk factors for dental and oral disease are the same risk factors for the occurrence of Non-communicable Diseases (PTM), which is the leading cause of death.

According to an internist, dr. Mangatas Manalu, Sp. PD., an anaerobic bacteria in the teeth and mouth can also be a source of infection to other body organs. Such as periodontitis caused by bacteria phymyromonas gingivalis, prevotella intermedia, and bacteriodes forsythus. The severity and increased occurrence of periodontitis can be increased in people with diabetes, and periodontitis also increases the risk of heart disease. So the problem on oral and dental health cannot be underestimated because based on data from WHO in 2015,
PTM causes 38 million deaths in the world every year. Before the discovery of a special antiseptic of the oral cavity, the way it is done is by mechanical means [2]. However, this is considered less effective in cleaning the mouth and teeth due to differences in the location of individual teeth. One more effective way is to use mouthwash, because of its ability to reach places that are difficult to clean by a toothbrush. However, antiseptics in mouthwash can have a carcinogenic effect on users [3]. The alternative to antibacterial and antiseptic that is naturally used is one of them using chitosan.

Chitosan is a chitin derivative natural polymer compound isolated from aquaculture waste, such as a crab shell crab which is the largest source of chitosan. In the health field, chitosan has the potential to serve as a wound dressing and help accelerate wound healing and blood clots. Chitosan has polycationic properties that can be used as an agglomeration agent. In addition, chitosan is also potential to serve as antimicrobials because it contains enzymes lysozyme and aminopolysaccharide groups that can inhibit the growth of microbes and is a hypoallergenic compound so little possibility of causing allergies. The ability to suppress bacterial growth in chitosan comes from the positively charged polycationic on chitosan capable of inhibiting the growth of bacteria and multicellular fungi [10].

Nanoparticles are particle sizes below 400 nm [12]. Nanokitosan can be absorbed by human organs that have nano-sized membrane penetration capabilities such as the kidneys, liver, and lungs. Therefore made a new breakthrough by modifying chitosan into nanoparticle-sized so that the effectiveness and application of chitosan can run more optimally.

2. Materials and Methods

Raw materials in this study using crab shell waste (Portunus pelagicus) obtained from the waters of Karangantu, Banten. Other materials used include HCl, NaOH, aquadest, and other chemicals for the analysis process.

The process of making chitosan through three stages, which is preceded by preparation, demineralisasi and deproteinas to get chitin, then do deacetylation to get chitosan.

2.1 Process of made Chitin

Waste shell crab is cleaned and dried. Screening of crumpled shells to obtain 80 mesh particle size. Demineralization initially soaked dried shrimp shrimp powder and dried shrimp with 1N HCl solution (ratio 1:7), leave for 120 hours. Furthermore, mereflux rendemen result for 1 hour at temperature 90°C, then filter the solids from the reflux product and washed until the neutral pH is then dried. The solids of the demineralization process are mixed with a 3 N NaOH solution (ratio 1:10), then silence for 24 hours. Then mereflux the result of immersion for 1 hour at temperature 90°C. Wash the filtered solid product with aquades until the pH becomes neutral and then dried to obtain chitin solids.

Table 1. Weight and rendemen of chitin and chitosan products.

| Initial Sample Weight (gr) | Product Weight (gr) | Rendemen (%) |
|----------------------------|--------------------|--------------|
| Chitin                     | Chitosan           | Chitin       | Chitosan     |
| 140                        | 28.6               | 14.8         | 20.43%       | 10.57%       |

2.2 Process of made Chitosan

Mixing chitin into 50% NaOH (b / v) solution at a ratio of 1: 10. Then reflux for 5 hours at 90°C. Next filter the chitosan that has direflux with filter paper. Washing chitin with filter results with aquades to neutral pH. Stretch back with the filter paper. Drying chitosan with oven at 40°C for 3 hours.

2.3 Process of made Chitosan Nanoparticles

Mixed 3 grams of chitosan and lactic acid solution 3% v/v 60 ml into beaker glass. Homogenize with magnetic stirrer for 2 hours. Adding aquades as much as 50 ml then dissolving NaTPP to reach concentration 0.1; 0.3; 0.5% w / v, then stirring using a magnetic stirrer for 20 min, until the soluble NaTPP solids. Filter remaining unsaturated solids with filter paper. Poured 50 ml of chitosan solution into the beaker, then complained using a magnetic stirrer at 350 rpm for 1 hour, followed by the addition of 10 ml of TPP solution with a concentration that has been slowly varied into chitosan solution, resulting in suspense nanoparticles.

2.4 Observation

The resulting chitosan then observed the characterization of functional groups using FTIR, particle size characterization using PSA. Measles deacetylation degree using acid-base titration method using 1% HCl and 1% NaOH. And conducted observations on blood clotting applications.
3. Results and Discussion

3.1 Chitin formation

The main mineral content in the crab shell is CaCO3 and Ca3 (PO4) 2 are smaller in number. The CaCO3 compound in the crab shell is more easily separated than the proteins because inorganic salts are only physically bound. Reactions that occur are:

\[
\text{CaCO}_3 (s) + 2\text{HCl} \rightarrow \text{CaCl}_2(l) + \text{H}_2\text{O}(l) + \text{CO}_2(g) \]

Based on these reactions, the appearance of CO2 bubbles generated in the demineralization process is an indicator of the reaction between HCl and CaCO3 mineral salts, the CO2 bubbles can be seen physically when adding HCl solution into the sample. Heating and stirring is done to facilitate the release of minerals from the crab shell and avoid overflowing air bubbles resulting from mineral separation. The washing process is performed to prevent degradation of the product when drying, in which the remaining HCl is able to break the chain of chitin compounds.

| Table 2. Chitosan grade [45]. |
|-----------------------------|
| **Grade Chitosan** | **%** |
| Low | 55-70 |
| Medium | 70-85 |
| High | 85-95 |
| Ultra High | 95-100 |

After going through the demineralization stage, the crabs are then depotinated using NaOH which is able to hydrolyze the protein into water-soluble sodium proteinate salt. Proteins in chitin cannot be removed entirely because the proteins in chitin form a stable complex. The efficiency of the deproteination process depends not only on the process conditions used (base concentration, temperature, time, and sample ratio by solution), but also depending on the species of the chitin source.

| Table 3. Characterization of Chitosan. |
|------------------|------------------|
| **Parameter** | **Commercial Chitosan** | **Chitosan Shrubs** |
| Water content | 2-10% | 8.5% |
| DDA | ≥70% | 80.5% |
| Solubility in acetic acid 2% | soluble | soluble |
| Size | Flakes to powder | 688.8 nm |
| Colour | White beige | White beige |

3.2 Chitosan formation

When mixing between chitin and NaOH solution, the OH-group addition occurs to eliminate the COCH3- group, which forms the NH2 group binding to the chitin polymer, called chitosan. The process conditions used are intended to break the bond between the acetyl group and the nitrogen atom so that it turns into an amino group (NH2). The stirring and heating process is carried out in order to accelerate the breaking of the acetyl group bond on the nitrogen atom, and to accelerate the replacement of the acetyl group with the amine group. A thick base solution is used because chitin cannot be removed entirely because the proteins in chitin form a stable complex. The efficiency of the deproteination process depends not only on the process conditions used (base concentration, temperature, time, and sample ratio by solution), but also depending on the species of the chitin source.

3.3 Characterization of Chitosan

Determination of degrees of deacetylation using the formula,

\[
\text{DD} = (\frac{\text{DD}^1 - \text{DD}^2}{M \times 0.00994} \times 0.016) \times 100\% \]

(2)
Based on the formula (2) obtained the calculation results obtained degrees of deacetylation of chitosan of 80.5% for crab shell. Result of chitosan characterization obtained from this research with chitosan national quality standard given in table 3.

3.4 FTIR characterization

The FTIR characterization results show a wide absorbs above 3100 cm\(^{-1}\), centered at 3151.69 cm\(^{-1}\) indicating a \(-OH\) supposedly covering peak \(-NH_2\) due to band formation in the same wave number region. In chitosan absorbance of amide I and amide II bonds were found at 1577.77 cm\(^{-1}\) and 1435.04 cm\(^{-1}\). FTIR results also show the role of acid in chitosan.

FTIR results also show the role of acid in chitosan, with FTIR showing similar absorbances to each other, this indicates the role of acid in the chitosan preparation reaction. Acid does not have intra- and intermolecular binding with di- and tri-carboxylic acids. So the acid only acts as a proton donor. The presence of C = O group in 1734.01 shows the presence of carboxylic acid which means acidity of chitosan particles increased. The emergence of N-H is caused by hydrogen bonding.

3.5 Characterization of particle size

The NaTPP solution used to minimize chitosan particles to nanometer size and make it in basic conditions, deprotonation and ionic cross linking between chitosan and NaTPP will occur. The hydroxyl ions and NaTPP will compete to react ionically with the \(-NH_3^+\) group in the chitosan by forming ionic glass bonds between chitosan and NaTPP and chitosan deprotonation reaction. Therefore, the pH of NaTPP solution is very influential in chitosan-TPP synthesis. Tripolyphosphate (TPP) is chosen as a crosslinker because NaTPP has more negative charge so it can interact more strongly than other polyanion such as sulfate and citrate. In addition, NaTPP is also non-toxic so it is not expected to change the biocompatibility of chitosan and is suitable for biomedical applications [47].

Concentration of NaTPP solution used 0.1; 0.3; and 0.5% as much as 10 ml for 1 hour with 350 rpm stirring. Stirring serves to get nanometer-sized chitosan. The longer the stirring will result in an increasingly smaller particle size as more and more particles are split into nanometer-sized particles [46]. This can be seen from the results of research in table 5.

Based on the results of chitosan particle size characterization showed that the best use of NaTPP solution is 0.1% concentration because the particle size is strongly influenced by the concentration and ratio of chitosan volume and NaTPP used, where the particle size increases with increasing concentration and volume ratio of chitosan and NaTPP [48]. Nano-sized particles can be obtained only with the use of NaTPP at very low concentrations and very small amounts. The concentration and the critical volume ratio of NaTPP that can be
used are 0.1% and 5: 1, if more than that it is easier to form heterogeneous nanoparticles measuring above 200 nm to micrometer. At 0.2% chitosan concentrations downward, the manufacture of nanoparticle-sized particles is relatively easier to do, wherein the effect of NaTPP concentration on the formation of microscopic particles is not very significant. The effect of NaTPP concentration is smaller with the lower chitosan concentration. This occurs because the amount of polycation of chitosan that will react with the polioanion of NaTPP is very small, so the formation of nanoparticles depends only on the concentration of chitosan.

### Table 5. Characterization of Chitosan size using particle size analyser.

| No | Name Sampel | Polidispersity Index | Ukuran (nm) |
|----|-------------|----------------------|-------------|
| 1  | NaTPP 0.1%  | 1.673                | 688         |
| 2  | NaTPP 0.3%  | 1.450                | 1.343       |
| 3  | NaTPP 0.5%  | 0.864                | 1.820       |

#### 3.6 Blood Clot Agent.

Nano Chitosan has biocompatible, biodegradable, non-toxic, antimicrobial properties that exhibit good biocompatibility and positive effects on wound healing. To help accelerate blood clotting, a simple hemostatis test was performed by clotting time (CT) tube method to determine the effectiveness of adding nanocitosan in stopping bleeding or as an agent to accelerate blood clotting. In the clotting time test, 1 mL of venous blood sample was inserted into the tube and added 2 drops (0.05 ml) of nanocitosan. The blood clotting time is calculated using a stopwatch and the tube is checked every 30 seconds until the blood is frozen. Clotting time (CT) blood test results with and without the addition of nanochitosan are shown in figure 2.

![Figure 2. Graph of clotting time of nanocitosan.](image)

#### Table 6. VWF levels: Ag & factor VIII C on ABO genotype.

| Genotype | Vwf : Ag (%) | Faktor VIII : C (%) |
|----------|--------------|---------------------|
| O/O      | 77.3 ± 27.4  | 131.8 ± 47.1        |
| A/O      | 113.8 ± 40.8 | 162.1 ± 52.5        |
| B/O      | 102.8 ± 30.2 | 155.5 ± 55.1        |
| A/A      | 118.0 ± 39.7 | 164.3 ± 50.5        |
| A/B      | 136.7 ± 33.7 | 170.9 ± 60.1        |

From Figure 2. it shows that nanocitoses with 0.5% NaTPP addition result in faster cloting time than the addition of NaTPP concentration at 0.1% and 0.3% concentrations, this proves that the larger the size of the nanochitosan particles used, the blood clotting process will take place faster. Nanocitosan is a hemostat that acts as a protrombin or procoagulant activator to help blood clots faster. Nanocytosan is gradually depolymerized to release N-acetyl-D-glukosamine, which initiates fibroblast poliferation, aids in providing collagen deposition orders and stimulates increased synthesis of natural hyaluronic acid levels at wound sites. This is what causes nanokitosan to help accelerate blood clots and wound healing and play a role in scar prevention [50, 51].

Time of clotting time is a measure of time used for hemostatic processes. The term hemostatis means the prevention of blood loss or control of bleeding when blood vessels are broken or broken. Hemostatis is performed by a variety of mechanisms namely vascular spasm, platelet formation, blood clots and fibrous tissue increments to seal the vents in a permanent vein [52].
The role of vWF in hemostasis is very important. Primary hemostasis is a mechanism to try to quickly block the vascular injury, by including endothelial, subendothelial, and platelet interactions. While factor VIII is a coagulant protein required for the activity of factor X on the intrinsic path [53].

Table 6. Shows that the vWF and factor VIII kadara in blood group O have lower levels than other blood groups, this causes the clotting time in blood group O to have a long time as seen from the results of blood clotting rate testing in Table 4.6 with the addition of nanocitoses, the hemostatic process in the wound may progress more quickly, can be seen from the comparison between negative control (without addition of nanocitosen) with the addition of nanochitosan, resulting in faster clotting time.

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