Flow Cytometry Detection of Bacterial Cell Entrapment within the Chitosan Hydrogel and Antibacterial Property of Extracted Chitosan

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ABSTRACT

***Background*:** Chitosan is unbranched polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine. Chitosan, derived from shrimp shell, has broad antimicrobial properties against Gram-negative, Gram-positive bacteria and fungi.

***Methods*:** Chitosan was extracted from shrimp shell and studied for cell entrapment and anti-bacterial properties. The hydrogel chitosan was used as the beads for cell entrapment and chitosan beads were designed to deliver cells and nutrients. These data confirmed with flow cytometric analyses.

***Results*:** Experimental results exhibited that internal diffusion through the chitosan matrix was the main mechanism for whole gelation by TPP (Tri-polyphosphate). The minimum inhibitory concentration (MIC) for chitosan against *Staphylococcus aureus* and *Escherichia coli* was 16 and 32 µg/ml respectively.

***Conclusion*:** Despite the antimicrobial properties of chitosan, trapped bacteria in the gel network were alive and were chelated indicating that their access to the outside was limited.

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Introduction

Chitin is a natural polysaccharide and is synthesized by enormous number of living organisms such as crustacean and fungi. Chemical structure of chitin is poly (β-(1→4)-N-acetyl-D-glucosamine) (Fig.1). This biopolymer after cellulose is the most abundant biopolymer in nature (1).

Chitin with an average degree of acetylation of less than 0.5 was called chitosan. This polymer is soluble in aqueous solutions containing organic acids such as acetic acid (2).

Chitosan is produced from chitin (under alkaline treatments), has wide applications in various fields. Chitosan is a random copolymer with a molar fraction DA (degree of acetylation) of β-(1→4)-N-acetyl-D-glucosamine and a fraction (1-DA) of β-(1→4)-D-glucosamine (1).

Chitosan has many well-known properties, including the non-toxicity, bio-functionality, biocompatibility and biodegradability that these properties are common among many polysaccharides.

In addition to the common properties, chitosan has unique properties such as film forming ability, antibacterial activity, absorption properties and bivalent minerals chelating ability (3).

In recent years, chitosan has been involved practically in dietary supplements, water treatment, removal of heavy metals, food preservation and packaging technology, tissue engineering applications, agriculture, cosmetics, pulp, paper and medical applications (5).

Chitosan nanoparticles are produced during interactions with negatively charged macromolecules. TPP has properties such as non-toxicity, polyvalent and ability of gels formation through ionic interactions therefore often used for the production of chitosan nanoparticles (6).

Semipermeable membrane to entrap living cells by using cell encapsulation method will be possible. This method is not only simple and applicable on a large number of cells, but also the trapped cells still retains its biological activity.

Encapsulation can inhibit cell surface antigens interact with components of the host immune system. In this method, diffusion of nutrients and metabolites from membrane easily possible (7).

This study aims to the formation of chitosan gel based on physical properties and mechanical stability. The formed gel was applied to entrap cells and analyze antibacterial activity of this polymer are against Gram-positive and Gram-negative bacteria.

Material and method

Extraction of chitin and chitosan

The Shrimp shells were obtained from Penaeus semisulcatus specimens collected from Local market. All the remaining loose tissues were isolated from shrimp shells and then rinse with cold tap water, dried in an oven about 24h at 50 °C.

After cooling the samples at room temperature, they were crushed into pieces about 0.5-5.0 mm. Chitin to chitosan conversation process consists of three main steps that include demineralization, deproteinization and deacetylation (8-10).

First, 25 g of sample was soaked in 1% hydrogen chloride and then was shacked for 24 h at room temperature to remove minerals. The remains of the sample were washed with deionized water and then drained off (9).

Sample was then treated with 50 ml of 3% sodium hydroxide for 1h to decompose the proteins into water soluble amino acids.

The remaining powder from these processes was boiled in a beaker containing 50% sodium hydroxide 2h at 100 °C and then the sample was cooled at room temperature for 30 min under the hood.

In order to remove sodium hydroxide from the sample, it was boiled in deionized water at 100 °C for 5min and it was then oven dried at 60 °C for 12h.

XRF analysis

The prepared chitosan powder was analyzed by XRF set (S4PINEER, Bruker, Germany). This analysis is able to detect the elements Fluorine to Uranium in the chitosan.

Chitosan gelation

Chitosan solutions were prepared by dissolving 0.05 g chitosan with sonication in 10cc acetic acid 1% solution until the solution was transparent.

Tripolyphosphate was dissolved in deionized water at the concentration 5 mg/ml. Equal volume of each solution were mixed together and formation of chitosan–TPP particles started spontaneously via the TPP initiated ionic gelation mechanism (11).

Assays for antibacterial activity

The minimum inhibitory concentration (MIC) of chitosan against *S. aureus*, *S. mutans*, *E. coli*, *P. stutzeri* and *S. agalactiae* were determined by the microdilution method, using Mueller-Hinton broth (MHB, Merck, Germany) and following the method described by the nature protocols (12).

In this method, Chitosan was diluted in a 96-well microtiter plate. Chitosan powder was accurately quantified and added to 1% acetic acid. The mixture was transferred to the second well and mixed with 60 µL 1% acetic acid, and similar transformations were repeated. Hence, each well contained a test sample solution with half of the concentration of the previous one. The wells were inoculated under aseptic conditions with 60 µL of the McFarland 0.5 standard prepared bacteria suspension. Finally, 60 µL of MHB was added to each well.

In order to ensure that the antimicrobial activity of chitosan is not related to 1% acetic acid, the entire process described above was repeated with 1% acetic acid.

The positive control was given with MHB and freshly prepared bacteria suspension, and the negative control wells were only contained MHB and 1% acetic acid. After mixing, the plates were incubated at 37 °C for 24h. The plates were then studied for the visible signs of growth or turbidity. The lowest concentration of chitosan that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC (13).

The minimal bactericidal concentration (MBC) was assessed as the extract concentration that gave significant MIC values after streaking the culture on Muller-Hinton Agar (MHA). Experiments were carried out in triplicate.

The cells entrapment

Overnight culture of *S. aureus* was prepared and then it was counted, 1ml of them was added to 1ml of the 0.5% chitosan solution. At the end 1ml 0.5% TPP solution was added to the mix. Tubes containing these materials leaved without shacking for 30 min at room temperature.

The number of bacteria remaining in the supernatant was evaluated by colony count method after 30 min gel formation and cell entrapment. This overnight bacteria and supernatant were analyzed by used for flow cytometry.

Sample preparation for flow cytometeric analysis

A stock solution of 42 µmol/L TO in dimethyl sulfoxide (DMSO) was prepared. A 10 times-diluted TO solution was prepared in DMSO just before staining of cells.

Prepared cell suspension of *S. aureus* (see above) was investigated for flow cytometeric analysis. 500 µL of samples were mixed with 5 µL of diluted TO solution and incubated at 30 °C for 5 min. Finally cells were washed with distilled water and re-suspended in distilled water (14).

Result

Demineralization, deproteinization and deacetylation

Mineralization and deacetylation processes didn't cause significant changes in sample weight but during the process of removing protein, weight was reduced from 25 g to about 15 g. Finally, approximately 15 g chitosan powder with white to creamy color was produced.

Antibacterial assessment

According to the literature, chitosan possess antimicrobial activity against a number of Gram-negative and Gram-positive bacteria. Table1 show the MIC and MBC of chitosan against various microorganisms in 1% acetic acid.

Table 1. MIC (µg/mL) and MBC (µg/mL) of chitosan solution.

Bacteria	MIC	MBC
E.coli	32	128
P. stutzeri	32	64
S.aureus	16	128
S.mutans	32	128
S. *agalactiae*	16	64

Trapping cell efficiency

Overnight cultures of *S. aureus* cells approximately 8.7 × 10^9 (cell/ml) were calculated. The number of cells remaining in the gel-bacteria supernatant, approximately 7.4 × 10^2 (cell/ml) were calculated. Figure 1 shows trapped bacteria cells in the chitosan-TPP network.

The interesting thing about trapped cells in the gel is that these cells were lived for about two months, although their number is reduced. Moreover, trapped bacteria in the gel network didn't die and just were chelated so their access to the outside was limited.

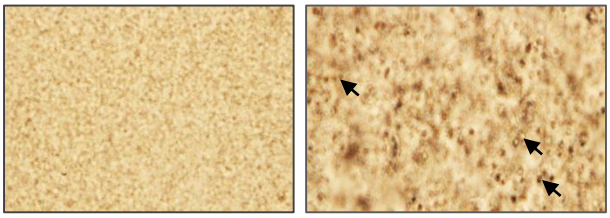

Figure 1. a) Chitosan gel matrix b) Entrapped *S. aureus* in chitosan gel network.

XRF analysis results

The XRF analysis was able to identify the elements Fluorine to Uranium in the samples that its accuracy is higher for heavy metals. According to the results of XRF analysis, this polymer has no heavy metals in its composition therefore the probability that the antimicrobial activity of chitosan was due to the presence of such elements, is less.

Interpretation of flow cytometeric analysis

In order to confirm the entrapment of bacterial cell within chitosan, Thiazole Orange (TO) (λex 509 nm; λ em 530 nm) was used. Briefly, TO is a permeant dye which enters freely in dead and alive cell, therefore it can clearly demonstrate the reaction of bacterial suspension before and after entrapment. As the figure 2 was shown the finger peak of bacterial suspension (Fig 2-a) apparently disappeared when bacteria entrapped in chitosan (Fig 2-b).

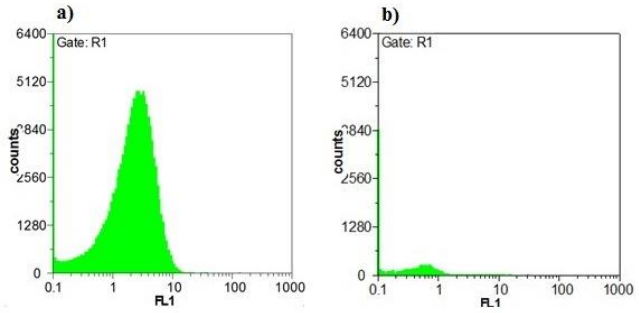

Figure 2. Flow cytometeric results of bacterial suspension a) before and b) after the entrapment in chitosan.

Discussion

Chitosan is a polysaccharide obtained acidic and alkaline treatments on shrimp shell which includes partial deacetylation of chitin (9). In this study, it can be concluded that chitosan production efficiency is about 60% which was higher than that reported by Hossain and Iqbal (16). This might be due to the longer duration of the process of deacetylation and deproteinization or the loss of some of sample during the washing process (15).

According to data obtained from this experiment, the antibacterial activities of chitosan against Gram-positive bacteria are higher than that of Gram-negative bacteria. In the past, many studies have been done on the antibacterial activity properties of chitosan, but in general the results suggest that chitosan antibacterial activity against Gram positive than Gram negative are more (16-18).

According to this findings and antibacterial properties of chitosan on the other hand, these findings can be interpreted such that the chitosan polymer has functional groups with antibacterial property and these functional groups during interaction with the TPP and gel formation, lose their antibacterial activity. Figure 3 shows a schematic of the functional groups involved in the interaction between the bacterial cell wall, chitosan and the TPP. Since the surface of chitosan gel contained functional groups, it is probably polymer network capable to trap other substances such as enzymes, proteins and drugs which can be used in the drug delivery and biotechnological aims.

Conclusion

As mentioned, chitosan is a biocompatible and biodegradable natural polymer with the ability to trap cells. With attention to all these positive properties, chitosan can be used to eliminate pathogens in contaminated water without harm to the aquatic ecosystem. Thus, shrimp shell which is an environmental waste used to clean up environmental contaminants.

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Conflict of interest

The authors report no conflicts of interest.

Financial disclosure

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