Antibacterial Activity of Chitosan Chemically Modified with New Technique

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The antibacterial activity of chitosan was chemically modified by introducing further amino groups to the back bone of chitin using parabenzoquinone (pBQ) as activation agent and ethylene di amine (EDA) as amino group source. The aminated chitin was further deacetylated to obtain finally chemically modified chitosan with higher content of amine groups. Factors affecting both activation process, and amination process amine grafting, have been studied through following its effect on the amount of introduced amine groups. The success of grafting process has been confirmed using FT-IR and TGA analysis. The antibacterial activity of the modified chitosan was tested on four different bacterial strains; two gram negative (Escherichia coli, pseudomonas aeruginosa), and two gram positive (Bacillus cereus, Staphylococcus aureus). It was found that the antibacterial activity of the modified chitosan is better than the native one, and increases by increasing the amount of introduced amine group (degree of the grafting), the degree of deacetylation. Lowering the molecular weight was found of negative effect. On the other hand, The Cytotoxicity activity test using Caco 2 cell line shows a safety result. As chitosan, modified chitosan show potential bactericidal activity along all strain examined spatially on the gram negative bacteria (Escherichia coli, pseudomonas aeruginosa). Finally, the modified chitosan shows higher solubility, almost double, at pH range from 5 to 6 comparing to chitosan it self. © Society for Biomaterials and Artificial Organs (India), 20080501-18.

Introduction

Microorganisms play an important role in our life it was dispersed in air, water and soil. Some of these organisms are human friendly and was useful in several fields, in the other hand, the other type was danger and may cause several problems in various fields.

In order to promote the general health and inhibit the infection of these bad organisms several materials was being employed as antimicrobial agents.

One of the most interesting substance is chitosan, chitosan that attract the attention of the scientist in last few years this because of its special properties including biodegradability, biocompatibility and non toxicity, it was a natural polysaccharide composed of linear poly β-(1-4)-2 deoxy-D glucopyranose (1-3). Chitosan has a broad rang application on several fields such as water treatment, spill oil removal, drug delivery, tissue engineering, wound healing, food preservation, enzyme immobilization.

Chitosan exhibits an antimicrobial activity against abroad range of microorganisms. There are several mechanisms which be suggested to explain this activity one of them...
was related this antimicrobial activity to the basic nature of the polymer and the amine content. Chitosan is a positively charged molecule and the target of its antimicrobial action is the negatively charged cell wall of bacteria, where it binds and disrupts the normal functions of the membrane, e.g. by promoting the leakage of intracellular components and also by inhibiting the transport of nutrients into the cells (4-6). The antimicrobial activity of chitosan is well known against a variety of bacteria and fungi coming from its polycationic nature (7). In another suggesting, chitosan was binded with DNA and inhibition of mRNA synthesis occurring via the penetration of chitosan into the nuclei of the microorganisms and interfering with the synthesis of mRNA and proteins in the final inhibit the bacterial growth. Furthermore, chitosan is a biomaterial widely used for effective delivery of many pharmaceuticals (8). Accordingly, chitosan may be suitable for incorporating other antipyretic for the preparation of long-acting antibacterial wound dressing.

The antimicrobial activity of chitosan increases with decreasing pH (5, 9-12). This is due to the fact that the amino groups of chitosan become ionized at pH below 6 and carry a positive charge. Unmodified chitosan is not antimicrobially active at pH 7, since it does not dissolve and also since it does not contain any positive charge on the amino groups (13, 14). The antimicrobial activity of chitosan also increases with increasing degree of deacetylation, due to the increasing number of ionisable amino groups (14). Several approached was done to increase the antimicrobial activity of chitosan by introduce amino group, on the primary amino groups of the back bone of the chitosan polymer chains but it was failed (15). The authors explained the obtained results to the position of the introduced amine groups.

In this work, we try to increase the antimicrobial activity of chitosan via increase the amino group on the polymer back bone by attaching amino group directly on the hydroxyl group of polysaccharide. A new technique has been used to avoid the consumption of the original amino groups of the chitosan as sites of grafting, so chitin was first grafted with amino groups in separate step, then it was deacetylated to have the aminated chitosan.

**Materials and Methods**

**Materials**

Sodium chloride (Purity 99.5%, M.wt.58.44) was obtained from Sigma-Aldrich Chemicals Ltd. (Germany). Acetic acid (Purity 99.8%, M.wt.60.05) was obtained from Sigma- Aldrich Chemicals Ltd. (Germany). Chitin from crab shells, practical grade was obtained from Sigma- Aldrich Chemicals Ltd. (Germany). Para Benzoquinone (Purity 99%, M.wt.108) was obtained from Sigma- Aldrich Chemicals Ltd. (Germany). Sodium hydroxide pellets (Purity 99-100 %, M.wt.40) was obtained from Sigma- Aldrich Chemicals Ltd. (Germany). Ethylene diamine (Purity 99%, M.wt.60) was obtained from Alfa asar, (Germany). Coomassie Brilliant blue G250, Ortho Phosphoric acid (Purity 85%, M.wt.98) ADWIC, (Egypt) Dehydrate alcohol (Purity 99.9%, M.wt.46.07). International co for supp&m. Industries, (Egypt) Tryptone powder Bacteriological Grade, Biobasic inc.(Canda). Agar bacteriological Grade, Gibcobri, (Scotland) YEAST EXTRACT, bacteriological Grade, Biobasic inc. (Canda).

**Microorganism**

Four bacteria were tested for the antimicrobial activity of chitosan and chitosan modified. these included two gram negative bacteria (Escherichia coli, pseudomonas aeruginosa) and anther two gram positive bacteria (Bacillus cereus, Staphylococcus aureus). Bacteria was incubated overnight at 37 °C in nutrient broth (peptone 1%, yeast extract 0.5 %, NaCl 1% and pH 5.5)

**Methods**

*Preparation of Modified chitosan*

The modification of chitosan was performed into three steps namely: chitin activation, amination and finally deacetylation.

In the first step, chitin activation, 4 gm of chitin was dispersed in 50 ml of distilled water at defined pH, dissolved in it pBQ and stirred for 6
hr. the activated chitin (AC) was separated and washed well with distilled water.

In the second step, chitin amination, the (AC) was dispersed in 50 ml of distilled water dissolved in it ethylene diamine and stirred for 6 hr. The aminated modified chitin (AMch) was separated and washed well with distilled water.

The last step, aminated chitin deacetylation, was performed according to Rigby and Wolfarn method (17, 18). The aminated chitin derivative was treated with 40 % aqueous solution of NaOH at 120-150 °C for 6 hr. The obtained aminated chitosan (AMC) was separated and washed well with distilled water.

Preparation of different MW of aminated chitosan

Aminated chitosan was degraded by the method of acetic acid hydrolyzes referenced from Chen et al (19). Aminated chitosan was dissolved in 5 % aqueous acetic acid incubated at 50 °C for 0, 3, 6, 16, 24 and 48 hr and then the centrifuged (5000 rpm) for 20 min. The supernatant was added to 4N aqueous NaOH. The sediment was filtrated and sequentially rinsed in water and ethanol and dried at 50 °C.

Characterization of modified chitosan

Qualitative determine of -NH₂ group, The Bradford assay (20)

This method is based on the observation that the Coomassie Brilliant Blue G-250 exists in two different color forms, red (365 nm) and blue (595 nm). The red form is converted to blue form upon binding of the dye to the auxochrome group –NH₂.

Changes in the amine groups content was monitored as follow: 100 µl of 1% of chitosan or modified chitosan solution in the acidic acid solution (2%w/v) was added to 900 µl of the Bradford reagent in a small test tube. The absorbance of the mixture was measured at 595 nm.

Infrared Spectrophotometric Analysis (FT-IR)

Analysis by FTIR Spectroscopic investigating structure for chitin, activated chitin, aminated chitin and modified chitosan were carried out using Fourier Transform Infrared Spectrophotometer (Shimadzu FTIR - 8400 S, Japan) to confirm occurrence of activation and amination reactions.

Thermal gravimetric Analysis (TGA)

Analysis by TGA of chitin, activated chitin, aminated chitin and modified chitosan were carried out using Thermogravimetric Analyzer (Shimadzu TGA –50, Japan) to evidence changes in structure as a result of activation and amination reactions.

Scanning Electron Microscopic Analysis (SEM)

Scanning of chitin, activated chitin, aminated chitin and modified chitosan were carried out using Analytical Scanning Electron Microscope (Joel Jsm 6360LA, Japan) to explore changes in the morphology resulted from activation and amination processes of the polymer matrix surface.

Solubility test

Solubility test of the sample was performed by dissolving a weighted sample in 2% acetic acid and stirred at room temperature at the desired time, and then the sample was filtrated, dried and weighted. The solubility was determined by the following equation (1):

\[
\text{Solubility} = \frac{\text{Weight of insoluble part}}{\text{Total weight of sample}} \times 100 \%
\]

Preparation of reagent

Coomassie Brilliant Blue G-250(100 mg) was dissolved in 50 ml 95% ethanol; to this solution 100 ml 85 % (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01 % (w/v) Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid.

Bio evaluation of modified chitosan

Antimicrobial activity (21, 22)

The measurement of the antimicrobial activity
of the chitosan and modified chitosan was done according to (21, 22). Briefly, the bacteria were inoculated in a L.B medium (1% peptone, 0.5% yeast extract and 1% NaCl) the inoculation was conducted at 37 °C for 24 hr with shaking the obtained bacterial suspension was diluted with the same peptone medium solution to 100 times.

0.1 ml of diluted bacteria suspension was cultured in 5 ml liquid peptone medium contain 0.1 ml of chitosan solution 1% has been sterilized under 121 °C for at least 20 min, the inoculated medium was maintained at 37 °C for 18 hr with shaking. The no of bacteria was counted by the ultraviolet absorbance of culture medium at 620 nm.

Bactericidal activity (23)

The bactericidal activity of chitosan and its derivatives were measured by enumeration of viable organism, the bacteria was growth in nutrient broth (peptone 1%, yeast extract 0.5%, NaCl 1%, pH 5.5) incubated overnight at 37 °C. the cultures obtained were diluted with autoclaved nutrient. One milliliter of the cell suspension was added to 1 ml of 1% chitosan derivatives that has been autoclaved at 121 °C for 20 min, the samples were removed after 0, 1, 2, 3, 4, 5, 6hr respectively. Portion were spread on triplicate nutrient agar plates, which were incubated at 37 °C for 24 h, and the numbers of colonies were counted.

Cytotoxicity test (24)

The evolution of the Cytotoxicity test was done with the direct contact method. The culture of mouse fibroblasts, with Ca 0.6 x 10^5 cells each were established on Petri-dishes on contact of the sample. After 24 hr the old culture medium is removed and a new one will added. The cell will be recorded after 120 hr and the viability will be calculated.

Results and Discussion

A- Preparation of modified chitosan

The structure of chitosan is useful to the synthetic chemist interested in site selective modifications. Such modifications have resulted into several derivatives of chitosan with distinct properties and applications. The presence of multiple nucleophilic groups within the chitosan backbone requires suitable synthetic protocol in order to obtain the desired selectivity. The synthetic transformation steps performed are often relatively simple, exploiting the difference in the nucleophilicities of primary amino group (at C-2) versus the two hydroxyl groups (at C-3 and C-6). From the literature it was found that the greater reactivity of amino groups rather than hydroxyl groups. However, the degree of selective substitutions varies greatly upon the reaction conditions. Several derivatives of chitosan was prepared to increase the amine content of chitosan via grafting it on the primary amino groups of the back bone of the chitosan polymer chains itself but the antibacterial activity of the product was decrease (15). In this work we will introduce external amino group to the chitosan on the hydroxyl groups rather than amino groups starting from chitin using para Benzoquinone (pBQ) as activating agent and Ethylene Di Amine (EDA) as source of the introduced amino groups. As shown in the following schema (figure 1)

Activation step

In this part we was activated the hydroxyl groups (C-3 and C-6) starting from chitin using pBQ.

Effect of the para Benzoquinone concentration

The effect of the pBQ concentration on the heterogeneous attraction of the hydroxyl group of chitin to the carbonyl group of the pBQ was investigated. The molar ratio of chitin relative to pBQ ranged from 1.1/16 to 1.2 (Figure 2). From the figure it was shown that increase the number of introduced amine groups by increase the molar ratio reached to 1:1/4 and then relatively stable by further increase of the molar ratio. The hydrophobic nature of chitin may be explained this trend which indicated occurring of the activation reaction only on the surface.

Effect of reaction’s pH on the activation step

The pH of the reaction medium of activation step was studied from 7.5 to 11 (figure 3). It was found noticeable increase of the potential of
the activation step by increasing the pH. The rises of the pH increase the electrophilicity of hydroxyl groups which attack the carbonyl group. The reaction is electrophilic substitution process which activated in the basic medium, and for this reason the authors ignore the acidic pH during the test.

**Effect of reaction’s temperature on the activation step**

The effect of the reaction temperature was studied from 20 to 60 °C. It was observed that there is no effect on the activation step by raising the temperature from 20 °C to the 40 °C. Slight increase at the 50 °C has been noticed. Again, these results paid our attention to the possibility of occurring the reaction on the surface of chitin due to its hydrophobic nature.

**Effect of reaction’s time on the activation step**

The effect of the time of the activation step was studied from 30 minute to 6 hr (figure 4). It was found increase the amine content on the polymer from 30 minute to the four hour and then leveling off. With further increase of reaction time. This trend could be referred to the consumption of hydroxyl groups on the surface of the activated chitin within four hours reaction time.

**Amination step**

In this step external amine groups were introduced to the structure of activated chitin (AC) by grafting ethylene di amine, as a source of amine groups, to the second carbonyl group of the pBQ as indicated in figure (1).

**Effect of ethylene di amine concentration on the amination step**

The effect of the molar ratio of the ethylene di amine relative to the para Benzoquinone attaché to activated chitin (AC) was studied in the range from 1:1/8 to 1: 2 (figure 5). It was observed a significant increase in the grafting of amine group by increasing the molar ratio till 1:1. This behavior could be attributed to increase the possibility of reaction between amine groups in the EDA and the carbonyl groups of the pBQ. Further increase of molar ratio over 1:1 leads to decrease the introduced amine groups. Cross linking ability of EDA between two pBQ activation centers could be an explanation to reduce the terminal induced amine groups.

**Effect of reaction’s temperature on the amination step**

The temperature profile of the reaction was studied in the range from 30 to 60 °C (figure 7). The amine content was found dependent on the reaction temperature, reversing to the activation step with pBQ, which the introduced amount increased gradually with temperature. Almost 40% of amine content increment has been obtained by increasing the temperature from 30 to 60°C. This behavior could be explained by the difference of the polymer matrix surface nature which is hydrophobic in the
activation step with pBQ and turns to be hydrophilic in the amination step. This hydrophilic nature of the surface affected by rising of temperature, during the reaction with EDA through increasing its rate of diffusion and consequently the rate of reaction with pBQ activated centers.

**Effect of reaction’s time on the amination step**

The reaction time was tested from 30 min to 4 hr (figure 6). It was found that increase the amine content of the polymer from 30 minute to the first hour and then decrease with further increase of reaction time. This trend could be referred to the consumption of most pBQ activated centers on the surface of the activated chitin in the first hour. Now the surface is crowded with introduced amine groups creating sterric hindrance and increasing the possibility of the reaction between the free terminal amine groups and the left pBQ free centers on the surface. This behavior leads finally to reduce the free terminal amine groups introduced to activated chitin.

**Table 1: the Effect of the degree of amination on the antimicrobial activity of modified chitosan against different microorganisms**

<table>
<thead>
<tr>
<th>pBQ : chitin ratio</th>
<th>Bradford absorbance</th>
<th>Maximum inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
</tr>
<tr>
<td>Chitosan control</td>
<td>0.095</td>
<td>0.626298</td>
</tr>
<tr>
<td>0.0935</td>
<td>0.105</td>
<td>0.774394</td>
</tr>
<tr>
<td>0.187</td>
<td>0.114</td>
<td>0.796616</td>
</tr>
<tr>
<td>0.374</td>
<td>0.251</td>
<td>0.831834</td>
</tr>
<tr>
<td>0.545</td>
<td>0.351</td>
<td>0.887889</td>
</tr>
<tr>
<td>0.747</td>
<td>0.355</td>
<td>0.907958</td>
</tr>
</tbody>
</table>

**Figure 2: Effect of pBQ concentration on the introduced amine groups reaction. (Reaction temperature 20°C and pH 10 for 6hr)**

**Figure 3: Effect of the pH of the activation step on the introduced amine groups reaction. (4mM of pBQ at 20 °C for 6 hr)**

**Figure 4: Effect of the activation process time on the introduced amine groups reaction (4mM of pBQ at 20 °C and pH 10)**
Antibacterial Activity of Chitosan

C. Evaluation of the antimicrobial activity of the modified chitosan

Amine groups of chitosan play an essential role on its antimicrobial activity. This role was observed as increase the potential inhibition of chitosan as increase the degree of deacetylation of it. The increase of the amino group substitution on the chitosan chains increases the positively cationic nature of chitosan in acidic solutions which lead to increase the chance to an interaction between the chitosan and the negatively charge on the cell walls of the microorganisms. This

Table 2: Effect of molecular weight of modified chitosan on its antimicrobial activity against different microorganisms

<table>
<thead>
<tr>
<th>Viscosity no</th>
<th>Maximum inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli</td>
</tr>
<tr>
<td>5.42262</td>
<td>0.863</td>
</tr>
<tr>
<td>7.744904</td>
<td>0.74</td>
</tr>
<tr>
<td>9.586797</td>
<td>0.713</td>
</tr>
<tr>
<td>15.3021</td>
<td>0.617</td>
</tr>
<tr>
<td>16.4881</td>
<td>0.482</td>
</tr>
<tr>
<td>17.0631</td>
<td>0.412</td>
</tr>
</tbody>
</table>

Table 3: Effect of the media pH on the antimicrobial activity of modified chitosan against different microorganisms

<table>
<thead>
<tr>
<th>Media pH</th>
<th>Maximum inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli</td>
</tr>
<tr>
<td>8</td>
<td>0.009554</td>
</tr>
<tr>
<td>7</td>
<td>0.100318</td>
</tr>
<tr>
<td>6</td>
<td>0.681529</td>
</tr>
<tr>
<td>5</td>
<td>0.762739</td>
</tr>
<tr>
<td>4</td>
<td>0.963232</td>
</tr>
</tbody>
</table>

Table 4: the solubility percent of the chitosan and amino chitosan in different pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Chitosan solubility</th>
<th>Modified chitosan solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>97.2</td>
<td>99</td>
</tr>
<tr>
<td>5</td>
<td>41.7</td>
<td>81.1</td>
</tr>
<tr>
<td>6</td>
<td>17.9</td>
<td>29.6</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>3.9</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Amide groups of chitosan play an essential role on its antimicrobial activity. This role was observed as increase the potential inhibition of chitosan as increase the degree of deacetylation of it. The increase of the amino group substitution on the chitosan chains increases the positively cationic nature of chitosan in acidic solutions which lead to increase the chance to an interaction between the chitosan and the negatively charge on the cell walls of the microorganisms. This

Figure 5: Effect of ethylene di amine concentration on the introduced amine groups reaction (30 °C for 6 hr)

Figure 6: Effect of reaction time on the introduced amine groups reaction. (1.8 mM of EDA at 30 °C)
observation stimulate Jukka Holappa (15) to increase the amine content of chitosan via grafting with quaternized betainates. But the new modified chitosan doesn’t give the required result. This was explained by the consumption of the more reactive amine group with another less reactive one. From this assay it was confirmed on the importance of the amine groups attached to backbone of the polymer chain on its antimicrobial activity.

Table 5: Effect of solvent type on antimicrobial activity of aminated chitosan against different microorganisms.

<table>
<thead>
<tr>
<th>solvent</th>
<th>Maximum inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli</td>
</tr>
<tr>
<td>ascorbic</td>
<td>0.73787</td>
</tr>
<tr>
<td>citric</td>
<td>0.919776</td>
</tr>
<tr>
<td>formic</td>
<td>0.964552</td>
</tr>
<tr>
<td>acetic</td>
<td>0.951493</td>
</tr>
</tbody>
</table>
Antibacterial Activity of Chitosan

Effect of the introduced amine groups amount on the antimicrobial activity

The degree of chitosan modification with external amine groups was studied (table 1). It was found that antimicrobial activity of the modified chitosan increased as a result of increases grafted amine groups. This leads consequently to increase the positive charges on the polymer. The selective grafting of this external amine groups on the hydroxyl groups of chitosan, increased its amine content in

Table 6: Bacteriocidal activity of chitosan against E. coli, Bacillus cereus, P. aeruginosa, and S. aureus

<table>
<thead>
<tr>
<th>Mixing time (hr)</th>
<th>E. coli</th>
<th>Bacillus</th>
<th>Pseudomonas</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>561</td>
<td>602</td>
<td>460</td>
<td>496</td>
</tr>
<tr>
<td>1</td>
<td>126</td>
<td>415</td>
<td>44</td>
<td>245</td>
</tr>
<tr>
<td>2</td>
<td>85</td>
<td>360</td>
<td>25</td>
<td>148</td>
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<tr>
<td>3</td>
<td>38</td>
<td>338</td>
<td>12</td>
<td>106</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>255</td>
<td>4</td>
<td>78</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>185</td>
<td>3</td>
<td>73</td>
</tr>
</tbody>
</table>

Table 7: Bactericidal activity of modified chitosan against E. coli, Bacillus cereus, P. aeruginosa, and S. aureus

<table>
<thead>
<tr>
<th>Mixing time (hr)</th>
<th>E. coli</th>
<th>Bacillus</th>
<th>Pseudomonas</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>494</td>
<td>373</td>
<td>263</td>
<td>451</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>102</td>
<td>33</td>
<td>145</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>63</td>
<td>26</td>
<td>106</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>25</td>
<td>7</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>21</td>
<td>6</td>
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<tr>
<td>5</td>
<td>1</td>
<td>20</td>
<td>4</td>
<td>37</td>
</tr>
</tbody>
</table>
addition to its effective original amine groups. In microbial profile, the powerful effect of modified chitosan on the gram negative rather than the gram positive bacterium was explained by the difference on the pathological composition of the cell wall.

Gram negative bacteria has thick layer of phospholipids rather than the peptidoglycan comparing to the gram positive which has thick layer of peptidoglycan. The negative charges of the phospholipids enhance the adhesion power of poly cationic polymer on the cell wall.

**Effect of the degree of deacetylation of the modified chitosan on its antimicrobial activity**

Effect of the degree of deacetylation of the aminated chitosan on its antimicrobial activity against gram negative and positive bacteria was studied (figure 8). It was found that the antimicrobial activity of the aminated chitosan increases with increase deacetylation time. This attributed to liberate more amine groups along the polymer chain which leading to increase the positive charges resulted from the protonation amino groups in acidic conditions.

**Effect of molecular weight of the modified chitosan on its antimicrobial activity**

Inhibition effect of the molecular weight of the aminated chitosan was studied on different bacterial species (table 2). The molecular weight of chitosan, as the amino groups, plays a great role in the antimicrobial activity. It was found that the antimicrobial activity effect of aminated chitosan decreased by decrease the molecular weight on both the gram positive and negative bacteria. In General, the decrease of the molecular weight of chitosan increases the antimicrobial activity but this increase was limited to certain molecular weight, after it the activity was declined.

This phenomenon was explained as decrease of the molecular weight of chitosan improves the movement of polymer chains in the solution by decrease the viscosity. Theoretically, Chitosan polymer was plugged the cell wall channels and inhibited normal metabolism of the cell. As far polymer molecular weight decreases, it escaped through the channel into the cell.

**Effect of the media pH on the antibacterial activity of modified chitosan**

The surrounding pH of the modified chitosan plays an essential role on its applications. This acidic pH responsible for solubility by forming the poly cationic character of the polymer. Based on this idea, the pH of the culture media was studied (table3). It was found that increase of the media pH decreased the antibacterial activity and this activity was limited in neutral and basic pH. This attributed to the solubility of the polymer. The solubility of chitosan and the

<table>
<thead>
<tr>
<th>sample</th>
<th>Live cells X 10^5</th>
<th>Dead cell X 10^5</th>
<th>Total cells X 10^5</th>
<th>viability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.75</td>
<td>1.2</td>
<td>7.95</td>
<td>89.9</td>
</tr>
<tr>
<td>Chitosan</td>
<td>5.35</td>
<td>0.8</td>
<td>6.15</td>
<td>86.9</td>
</tr>
<tr>
<td>aminated Chitosan</td>
<td>5</td>
<td>1.5</td>
<td>6.5</td>
<td>76.3</td>
</tr>
</tbody>
</table>
aminated chitosan was measured as in table 4. The improving of the solubility of aminated chitosan over chitosan itself was attributed to grafted amino groups. The aminated chitosan solubility at pH 5.0 to 6.0 is almost double of its unmodified chitosan counterpart.

**Effect of the type of amine on the amination process of chitosan**

The effect of the separation distance between the amine group and the backbone of the polymer on the antimicrobial activity was studied figure (9). In this way different type of the aminated chitosan was prepared using ethylene di amine and hexamethylene di amine as sources of the induced amine groups. From the figure it was found that the increase the reactivity from chitosan to the modified chitosan (using ethylene di amine) which direct result to increase the amine groups along the polymer chain. By replacing the ethylene di amine with hexamethylene di amine the antimicrobial activity decrease slightly as a result of increase the distance, the chain polymer backbone this behavior could not be referred to the amount of introduce amine groups science it has not be affected.

This study showed that increase the distance between the amine groups and the backbone (in case of hexamethylene di amine) decreased the effectivity of the amine group. These results represent the role of the pyranose ring on the mechanism of action. It is also confirmed the powerful activity of the original amine groups which attached directly on the chain’s backbone over other amine groups. In addition, it explains the decrease of the antibacterial activity of the N-grafted chitosan because these modifications replaced the more active amine groups with less active ones.

**Effect of solvent type on the antibacterial activity of modified chitosan**

The effect of the aminated chitosan solvent on its antimicrobial activity was studied using four different organic acids namely acetic, formic, citric and L-ascorbic acid (table 5). It was found that the potential of the activity was decreased in order formic acid > acetic acid > citric acid >> L-ascorbic acid. Increase the potential of the formic rather than acetic at the same concentration attributed to decrease the molecular weight and size which enable it to separate the polymer chain and increased its solubility other than acetic acid. In the other hand the bivalent character of citric acid decrease its solubility in the case of L. ascorbic acid it enhance the metabolism of the microorganism and soften the effect of potential activity of chitosan.

**Bactericidal activity of modified chitosan**

The bactericidal activity of chitosan and aminated chitosan was studied on gram negative and gram positive, table 6 and 7. The decreases of the number colonies by time indicate that they are not only stopping the growth of the bacteria but also killed it. From tables it was observed a higher killing potential of aminated chitosan over chitosan itself according to the rate of declining of the colonies number.

It was found that increase the inhibition of gram negative rather than gram positive bacteria which attributed to the physiological structure of difference the cell wall of the two strains.

This result confirmed the rapture of the cell wall mechanism rather than nuclear protein interaction mechanism. The interaction of the amine groups of modified chitosan with the cell wall decrease its selective permeability which leads to leakage of the intracellular substance, such as electrolytes, UV-absorbing material, protein, amino acids, glucose, and lactic dehydrogenase. As a result, chitosan and modified chitosan inhibit the normal metabolism of microorganisms and finally lead to death of this cell.

**Cytotoxicity test of modified chitosan**

Cytotoxicity of Chitosan and aminated Chitosan was tested using caco 2 cell line with the direct connect method and the data are shown in table 8.

Several search was tested and measured the cytotoxicity of Chitosan. Although Chitosan exhibits some concentration dependent
cytotoxicity at high degree of deacetylation, this value still in the safe area. In the following test the viability of the live cell was decreased by increase the amine group substitution, however, the toxicity of aminated chitosan is negligible.

Table 8: Cytotoxicity result of chitosan and modified chitosan. (0.6 x 105 cells incubated for five days)

Polymer characterization

FT-IR analysis

The FT-IR spectrum of the modified chitosan and intermediates were obtained using FTIR-8400S SHIMDZU Japan. As shown in figure 10, the major difference are the wide peaks at 3431 cm-1, (I) corresponding to the stretching vibration of –NH-2 and OH groups become more sharp at modified chitosan result from substitution of –OH groups with –NH2 groups.

Absorption band intensity at 1560, 1649 cm-1 (II) corresponding to amide bands have been increased in (AC )via introduce further carbonyl groups of pBQ (curve b) then return to normal intensity at aminated chitin (curve c) as a result of reaction the carbonyl groups with amine groups of EDA during amination step. Finally, peaks have been reduced after deacetylation as a result of removal the acetyl groups in modified chitosan (curve d).

Scan electron microscope (SEM micrograph)

As we observed in the SEM micrographs in (figure 11), the surface of the chitin become rougher after amination. This attributed to grafting process on the surface hydroxyl groups of chitin particles. Also an increase of aminated chitosan roughness than chitosan has been observed. The increase of the surface roughness is automatically combined by increase of the surface area which enhanced its adhesion with microorganism cell wall.

TGA analysis

Thermal Gravimetric Analysis (TGA) of modified chitosan and the intermediate products were carried out using TGA-50 SHIMADZU Japan. As shown in figure 12, the first weight loss at temperature 118 °C result from the evaporation of the water from the sample indicating the increase of the water content in the samples 9.459, 9.939, 10.384, 12.248% of chitin and activated chitin, aminated chitin and modified chitosan respectively. This confirmed the increase of the hydrophilic properties of chitin through modifications. The second weight loss of samples was start at 256 °C. The decrease of the weight loss percent of the modified chitosan rather than chitin could be attributed to the introduced amine groups.

Conclusion

The antibacterial activity of chitosan was increased by grafting amino groups to its backbone chains. It was found that the antimicrobial activity of chitosan was dependant on the degree of grafting, degree of deacetylation, the molecular weight and the pH of the tested media. The modified chitosan has stronger effect on the gram negative bacterial rather than the gram positive one. Also the bactericidal activity was shifted to right by introducing amino groups. In conclusion, the introducing of more amino groups directly on the backbone of chitosan, in addition to its original amine groups has successed in increasing its antibacterial activity and doesn’t affect its cytotoxicity.

Reference