Specific effects of calcium acetate on the self-assembly of chitosan molecules on mica surface observed with atomic force microscopy

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ABSTRACT
The well-organized chitosan film on mica surface has drawn wide attention for its potential applications and studies on the process of self-assembly. In this article, atomic force microscopy was used to investigate the ultrastructure of the self-assembly of chitosan molecules with calcium ions in different concentrations. It was showed that calcium ions might affect the self-assembly of chitosan to a certain extent, and chitosan molecules gathered from fibrils to circular, and finally formed to film with the increasing concentration of calcium ions. Further, the formation mechanism of Ca(II)-chitosan conjugates was adapted to explain the effects of calcium ions on the self-assembly of chitosan. These results and the related analysis would be valuable for guiding the pattern of chitosan self-assembly with a potential application in food packaging science and engineering.

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Introduction
In recent years, as people pay more attention to food safety, the requirements of non-toxic and biodegradable material are becoming increasingly urgent. Chitosan is a unique alkaline polysaccharide in the nature obtained by partial or total removal of acetyl groups from chitin. Chitosan is not only abundant and biodegradable but also a renewable resource. The chitosan is easy to form film which has a certain permeability of $O_2$, $CO_2$, and water vapour. It has many valuable properties such as biocompatibility, antibacterial property, biodegradability, low toxicity, and metal binding ability.[1–5] Moreover, antibacterial property makes the film has a broad prospective. Over the past two decades, the performance of chitosan film has been reported extensively.[6,7] Although chitosan film in food preservation has a great advantage, the film has high water vapor permeability, poor mechanical strength, narrow antibacterial range and other shortcomings, which limit its application scope. Since the chitosan molecule contains many amino groups and hydroxyl groups in each unit, it could be complexed with metal ions and has strong intramolecular hydrogen bond.[8–10] Therefore, metal ions act as a cross-linking agent, making the similar reticular structures formed by hydrogen bonds or salt bonds between chitosan molecules more compact.

Due to the good coordination of chitosan with metal ions, it presents many special physical and chemical properties as well as biological activity, so it has been widely applied in chemical industry, pharmaceutical industry, agriculture, bioengineering, food industry, and so forth.[11] There were many studies on the adsorption of chitosan and $Fe^{2+}$, $Cd^{2+}$, $Pb^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Ag^+$ conjugates.[12–16] Unfortunately, very little attention has been paid to the complexation between chitosan and $Ca^{2+}$.

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The related results showed that, chitosan mainly reacts with a calcium ion through its four amino,[17] and the schematic diagram of complexation mechanism was shown in Figure 1. While many studies have shown that, Ca(II)-chitosan conjugates film have a certain preservation effect on mango, fresh lettuce, pumpkin, carrot, strawberry, and so on.[18–22] Moreover, calcium is a better choice to apply to the food preservation as a constant element of human body. Therefore, Ca(II)-chitosan conjugates film is worthy of study.

Previous studies have shown that, chitosan aggregates in star-like structure, while those which absorb Ag⁺ grow disc-like structure and the Ag(I), Cu(II)-chitosan conjugates fractal dimension is smaller.[23] It could be speculated that adding metal ions can regulate the morphology of the film. At the same time, the atomic force microscopy (AFM) that we chose to study self-assembly was an efficient and widely used technique in molecular and cellular biology for its nano-scale resolution and simple sample preparation methods. Many AFM studies indicated that it could acquire lots of information on the self-assembly of chitosan on mica surface.[24–30] Thus, atomic force microscopy (AFM) was used to investigate chitosan and Ca(II) conjugates with different concentrations in this work.

### Materials and methods

**Preparation of chitosan solution**

Totally, 5 mg/mL chitosan was obtained by dissolving chitosan with the deacetylation degree of 80.0%–95.0% (Guiyang Chemical Co., Guizhou, China) using 1% acetic acid solution (Purity ≥ 99.5%, Guanghua Chemical Factory Co., Guangdong, China), and then to configure the concentration of 5 mg/mL chitosan, and then diluted to 100 μg/mL, 200 μg/mL, 500 μg/mL, 1 mg/mL, 2 mg/mL, and 3 mg/mL separately. After standing for a while, 10 μL of the solutions were added onto the freshly cleaved mica sheets (Tosai, Hong Kong, China) in 1.0 × 0.5 cm², finally were placed, covered and dried in 12-wells plates at room temperature.

100 mM CaAc₂ (Chengdu Kelong Chemical Reagent Factory Chengdu, China) was prepared with 1% acetic acid solution, and it was diluted to prepare CaAc₂ solution that was twice the concentration of the desired solution at concentrations of 0.2 mM, 0.4 mM, 1 mM, 2 mM, 4 mM,

![Figure 1. The schematic diagram of the complexation mechanism of Ca(II)-chitosan conjugates.](image-url)
10 mM, 20 mM, 40 mM, 100 mM. Then 100 μg/mL chitosan and CaAc$_2$ solution were mixed up in equal volume to get Ca(II)-chitosan conjugates, then was placed, covered and dried in 12-wells plates at room temperature.

**AFM imaging of the self-assembly of chitosan**

A Multimode-8 type AFM (Bruker Co., Santa Barbara, USA) was used in ScanAsyst mode with ScanAsyst-AIR probes to image the naturally dried chitosan at 0.977 Hz. AFM was used for visualization of chitosan and Ca(II)-chitosan conjugates deposited on mica sheet. First placed the sample on the stage, adjusted the light mirror to find a clean location under the light microscope, then placed the needle holder and fixed it so that the needle was just touching the sample. First selected the 500 nm scan range, align the X-axis and Y-axis to the origin position. Finally chose typical region to gain images when the range was extended from 500 nm, 1 μm, 2 μm, 5 μm, to 10 μm. All images are typically obtained from at least five macroscopically separated regions on each sample at room temperature with the related humidity of 55%.

**Data analysis**

Typical height images, roughness, and surface area difference were statistical analyzed using the offline software Nanoscope Analysis V1.5 (Bruker Co., Santa Barbara, USA) after using 1st order flatten and noise-line eraser to smooth the image. Then, the “section” function could output the section profiles. And root mean square roughness (Rq) and average roughness (Ra) were collected by the “roughness” function to describe the topological change, and the surface area difference was gathered to indicate the degree of surface coverage. All data were expressed as mean ± standard deviations. Analyses were performed using Excel 2010 (Microsoft Co., Redmond, USA) and SPSS22.0 (IBM Co., Armonk, USA).

**Results and discussion**

**Self-assembly of chitosan in different chitosan concentration**

Firstly, the effect of chitosan concentration on the structure of the assembly on mica surface was investigated by AFM and the typical height images and section profiles curves were collected and shown in Figure 2. The values of height, roughness (Ra, Rq) and surface area difference were shown in Table 1. When the concentration of chitosan is low, chitosan was uniformly dispersed on the mica sheet (Figure 2a-c). At 0.01 mg/mL, the height was 1.07 ± 0.21 nm, Ra was 0.153 nm, Rq was 0.251 nm, surface area difference was 0.114%. While the height of 0.01 mg/mL chitosan was increased to 4 ± 2, and Ra was 0.214 nm, Rq was 0.430 nm, surface area difference was 0.115%. With the concentration increased slowly to 0.05 mg/mL (Figure 2c), the height was decreased to 0.90 ± 0.26 nm, Ra was 0.271 nm, Rq was 0.343 nm, surface area difference was 0.111%, the chitosan particles became more intensive and began to form a little short, thick fibers.

As shown in Figure 2d-f, when the concentration of chitosan increased from 0.1 mM to 0.5 mM (Figure 2d-f), the height changed from 8.59 ± 0.11 nm, 11.43 ± 0.26 nm, to 8.11 ± 0.19 nm in turn, Ra was 2.02 nm, 1.94 nm, and 2.24 nm, successively, Rq was 2.52 nm, 2.47 nm and 2.73 nm in turn, and surface area difference varied from 1.07%, 1.41%, to 1.70% one by one. Chitosan particles were evenly and densely distributed on the surface of mica at 0.1 mg/mL, as shown in Figure 2d. With the increase of concentration, partial chitosan fibers firstly gathered due to the interaction among chitosan molecules. At 0.2 mg/mL (see Figure 2e), the average vertical height of chitosan particles increased to 11.43 ± 0.26 nm, compared with the data of chitosan at 0.1 mg/mL. However, the Ra and Rq did not change significantly. While the chitosan concentration further increased, chitosan fibrils were largely formed and began to overspread in the whole picture for the enhanced interaction among chitosan molecules. Thus, the height
Figure 2. Typical AFM height images of chitosan assembly on mica surface at different concentrations. (a) 0.01 mg/mL, (b) 0.02 mg/mL, (c) 0.05 mg/mL, (d) 0.1 mg/mL, (e) 0.2 mg/mL, (f) 0.5 mg/mL, (g) 1 mg/mL, (h) 2 mg/mL, and (i) 3 mg/mL.
of 0.5 mg/mL chitosan (Figure 2f) was reduced to 8.11 ± 0.19 nm, but Ra, Rq and surface area difference were increased simultaneously. Chitosan fibrils became dense obviously and distributed evenly at 1 mg/mL (Figure 2g). The height of the 1 mg/mL chitosan was reduced more than a half to 3.31 ± 0.10 nm, Ra was 1.09 nm, Rq was 1.33 nm, and the surface area difference was considerably reduced to 0.593%. When the chitosan concentration increased to 2 mg/mL (Figure 2h), rough chitosan film was formed with the height of 12.20 ± 0.75 nm, Ra and Rq increased to 3.43 nm, and 4.35 nm individually, and the surface area difference was 1.91%. After the concentration of chitosan increased to 3 mg/mL (Figure 2i), the chitosan film got thicker with the chitosan particles height of 16.84 ± 0.61 nm, Ra of 3.40 nm, Rq of 4.24 nm, and surface area difference of 1.14% separately. Although the height increased, the Ra and Rq changed little compared to the data of chitosan at 2 mg/mL.

It was found that chitosan was gathered from short and thick fibers (Figure 2d) to long and fine fibrous structure (Figure 2e), and the number of chitosan fibrils obviously increased with the increase of chitosan concentration (Figure 2f-g). The intermolecular and intramolecular hydrogen bond played a key role in chain gathering. Finally, rough and dense chitosan film was formed (Figure 2h-i).

**Self-assembly of ca(ii)-chitosan conjugates in different caac2 concentration**

Because 50 μg/mL chitosan fibers were evenly distributed, and its shape was clear, to farther gain the clear pattern and mechanism of interaction between calcium ions and chitosan molecules in details, the chitosan concentration was reduced to 50 μg/mL in the further study. To investigate the effect of calcium ions concentration on the self-assembly of chitosan molecules, calcium acetate was added to the anion interference. Chitosan molecules assembled on mica surfaces with increasing concentration of calcium ions and the AFM height images were shown in Figure 3. The Values of height, roughness (Ra, Rq) and surface area difference derived from the AFM height images were shown in Table 2. The line chart of height, Ra, Rq change with concentration were shown in Figure 4.

**Table 1.** Height, roughness (Ra, Rq) and surface area difference of the self-assembly of chitosan molecules in different chitosan concentrations.

| Concentration of chitosan (mg/ml) | Height (nm) | Ra (nm) | Rq (nm) | Surface Area Difference |
|----------------------------------|-------------|---------|---------|------------------------|
| 0.01                             | 1.07 ± 0.21 | 0.153   | 0.251   | 0.114(%)               |
| 0.02                             | 4 ± 2       | 0.214   | 0.43    | 0.115(%)               |
| 0.05                             | 0.90 ± 0.26 | 0.271   | 0.343   | 0.111(%)               |
| 0.1                              | 8.59 ± 0.11 | 2.02    | 2.52    | 1.07(%)                |
| 0.2                              | 11.43 ± 0.26| 1.94    | 2.47    | 1.41(%)                |
| 0.5                              | 8.11 ± 0.19 | 2.24    | 2.73    | 1.70(%)                |
| 1                                | 3.31 ± 0.10 | 1.09    | 1.33    | 0.59(%)                |
| 2                                | 12.20 ± 0.75| 3.43    | 4.35    | 1.91(%)                |
| 3                                | 16.84 ± 0.61| 3.40    | 4.24    | 1.14(%)                |

**Table 2.** Height, roughness (Ra, Rq) and surface area difference of the self-assembly of 50 μg/mL chitosan molecules in different CaAc2 concentrations.

| Concentration of CaAc2 (mM) | Height (nm) | Ra (nm) | Rq (nm) | Surface Area Difference |
|-----------------------------|-------------|---------|---------|------------------------|
| 0.1                         | 2 ± 1       | 0.549   | 0.432   | 0.130(%)               |
| 0.2                         | 1.95 ± 0.98 | 0.478   | 0.606   | 0.172(%)               |
| 0.5                         | 3.30 ± 1.59 | 0.721   | 0.898   | 0.312(%)               |
| 1                           | 3.80 ± 0.92 | 0.993   | 1.13    | 0.228(%)               |
| 2                           | 5 ± 1       | 2.84    | 3.76    | 1.32(%)                |
| 5                           | 7 ± 3       | 4.79    | 5.93    | 1.40(%)                |
| 10                          | 10 ± 2      | 3.01    | 3.81    | 0.427(%)               |
| 20                          | 20 ± 6      | 3.20    | 4.08    | 0.915(%)               |
| 50                          | 62 ± 5      | 4.27    | 3.98    | 0.482(%)               |
Figure 3. Typical AFM height images of 50 μg/mL chitosan assembled on mica surface in different concentration of CaAc₂. (a) 0.1 mM, (b) 0.2 mM, (c) 0.5 mM, (d) 1 mM, (e) 2 mM, (f) 5 mM, (g) 10 mM, (h) 20 mM, and (i) 50 mM.
It can be seen from the AFM height images that the morphology of self-assembled Ca(II)-chitosan conjugates was largely different in the presence of different CaAc\textsubscript{2} concentrations. In the presence of 0.1 mM CaAc\textsubscript{2}, particles gathered closely with height of 2 ± 1 nm, and Ra of 0.549 nm, Rq of 0.432 nm, and surface area difference of 0.130%, as shown in Figure 3a. With the increase in calcium ions concentration, chitosan particles gathered into fibers and mutually crosslinked on the mica sheet. In the presence of 0.2 mM CaAc\textsubscript{2}, fibril-like structure of assembled chitosan was observed in Figure 3b. At 0.5 mM CaAc\textsubscript{2}, chitosan fibrils were much thicker than that in 0.2 mM CaAc\textsubscript{2}, as shown in Figure 3c. While the CaAc\textsubscript{2} concentration further increased to 1 mM (Figure 3d), the fibers became more rough and denser. Then the numbers of chitosan fibers were significantly increased, and fibers became largely thick. At 2 mM CaAc\textsubscript{2} (Figure 3e), the fibers distributed very close, and the height tended to be evenly. While at 5 mM CaAc\textsubscript{2} (Figure 3f), the fibers start to further crosslink based on the multilayered fibers furthermore. The concentration of calcium ions increased from 0.2 mM to 5 mM (Figure 3b-f) and the height changed from 1.95 ± 0.98 nm, 3 ± 2 nm, 3.80 ± 0.92 nm, 5 ± 1 nm, to 7 ± 3 nm in turn, the Ra values grew from 0.478 nm, 0.721 nm, 0.993 nm, 2.84 nm to 4.79 nm, the Rq values increased from 0.606 nm, 0.898 nm, 1.13 nm, 3.76 nm to 5.93 nm, and the surface area difference in turn was 0.172%, 0.312%, 1.32%, and 1.40%. And the data shows an increase in regularity. At 10 mM CaAc\textsubscript{2}, fibers formed into the ring-like structure due to fibers continuously crosslinked and gathered with the height of 10 ± 2 nm, and Ra of 3.01 nm, Rq of 3.81 nm, and surface area difference was reduced to 0.427%, as shown in Figure 3g. The white part of the figure showed where fibers tended to gather. And the size of the ring depends on how many crosslinked fibers gathered locally. It was shown that in Figure 3h, the fibers gathered and resulted in a long-range orderly effect. The main agent of the formation of structure originated from the surface tension and anisotropy. The ring-like structure of chitosan assembly was formed into film at 20 mM CaAc\textsubscript{2} with the height of 20 ± 6 nm, Ra of 3.20 nm, Rq of 4.08 nm, and the surface area difference of 0.915%. Finally, at 50 mM CaAc\textsubscript{2}, the film’s height increased by three times to 62 ± 5 nm, Ra increased to 4.27 nm, Rq increased to 3.98 nm, and surface area difference also increased to 0.482% simultaneously. It was obvious that, the Ra and Rq did not change much (Figure 3i).

With the increase in CaAc\textsubscript{2} concentration, due to hydrogen bonding and intermolecular forces, chitosan firstly assembled on mica surfaces from the point to the fibrils (Figure 3a-b), and then chitosan fibrils grew to more intensive fibers (Figure 3c-d). Then the fibers gathered further thicker (Figure 3e-f), then gathered into circular (Figure 3g), and finally the ring-like structure of chitosan assembly gathered into the film (Figure 3h-i). The height was close to linear growth, the Ra, Rq, and surface area difference increased slowly with it. The fitting function was showed in Figure 4. It was

\[ y = 1.1679x + 1.2598 \]

![Figure 4](image_url). The line chart of height and Ra change with concentration.
shown that calcium ions will affect the self-assembly of chitosan to a certain extent, and the results indicated that Ca(II)-chitosan conjugates are easier to form fibrils and film.

**Conclusion**

In summary, it was found that the self-assembly of chitosan and Ca(II)-chitosan conjugates is a dynamic process. During this process, calcium ions improve chitosan ability to enhanced interaction. Compared to single chitosan self-assembly, chitosan assembled on mica surface in the presence of CaAc$_2$ easily tends to crosslink and gather into film. With the increase in CaAc$_2$ concentration, chitosan firstly assembled on mica surfaces from the particles to the fibrils, then chitosan fibrils became more intensive fibers. Then the fibers gathered further thicker, then gathered into circular, and finally gathered into the film. The film derived from 50 μg/mL chitosan in the presence of 20 mM CaAc$_2$ become obvious thick and smooth. When the concentration of calcium ion was increased to 50 μg/mL, the Ca(II)-chitosan film can be very thick. In contrast, single component chitosan film is not smooth enough. Based on the theoretical basis of chitosan complexation with calcium ion, the change of chitosan self-assembly in the presence of different calcium ion concentration can be controlled according to the demand. It may provide theoretical guidance and data supports for the research of the structural stability of Ca(II)-chitosan conjugates and its physiological and nutritional effects such as calcium supplementation.

**Declaration of interest**

The authors declare that they have no conflicts of interest.

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