doi:10.1246/cl.2010.935
Published on the web July 24, 2010

Preparation of Dispersible Chitosan Particles with Borate Crosslinking for Antimicrobial and Antifungal Application

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This paper introduces a facile preparation method using chitosan lactate with borate crosslinking for creation of dispersible chitosan submicron spheres and its excellent antimicrobial and antifungal activities.

Chitosan as a cationic biopolymer has been deployed for various applications due to avirulence, biocompatibility, biodegradability, etc.[1] For example, in biomedicine, items in which chitosan and its derivatives have been incorporated are microparticles and are used as packings for chromatography,[2] carriers for enzyme immobilization,[3] affinity adsorbents,[4] endotoxin adsorbents,[5] drug carriers,[6] elicitors for plants and antimicrobial agents in pesticides or food products.[7] Among these, an application that has been attracting particular attention recently is its use as an antimicrobial agent that is biofriendly, but its range of use is limited because of the fact that the antimicrobial activity is restricted to acidic conditions since the electric charge is lost at a pH of 6.5 or above and the fact that its solubility is remarkably degraded in the neutral region due to the production of strong hydrogen bonds.[8] The use of dispersible chitosan particles has been considered as a method to solve these kinds of chitosan problems. For example, the suspension evaporation method is known for preparation of spherical microparticles,[5] but a submicron size particulation, which is thought to be more highly dispersible, has not been achieved. In addition, a particulation method using spray drying is also known,[9] but the control of the particle diameter is difficult with this method and control at 10 μm or less is particularly difficult.

In this paper, a new and facile method for the production of dispersible particles of chitosan from several microns to submicron size will be introduced. This method is one in which boric acid forms a complex with a sugar chain and particulation is done by a crosslinking reaction.[10] This method does not require heating and, moreover, does not use any surfactant or organic solvent, which are burdens on the environment. In addition, the chitosan particles that are obtained exhibit superior antimicrobial activity toward not only bacteria but also fungi even near neutrality.

The chitosan–boric acid complex particles (CB-*X*) were prepared by the addition of an aqueous solution of sodium metaborate to a lactic acid solution of chitosan.[14] The amount of added boric acid was adjusted such that it became 0.5, 1.0, and 2.0 equivalent (*X*) per unit of glucosamine in the chitosan, and three types of complex particles (CB-0.5, CB-1.0, and CB-2.0) were produced in this study. It was possible to track the production of the particles from the chitosan solution by the fact that the solution becomes cloudy together with the addition of the sodium metaborate. Figure 1 is the UV–visible spectrum one hour after the addition, and it shows that the transmittance becomes lower (the cloudiness increases) the greater the amount of sodium metaborate added. Figure 1 shows that even after the lactic acid and metaboric acid that are present in excess have been removed by dialysis, a low degree of cloudiness is maintained resulting from the particulation.

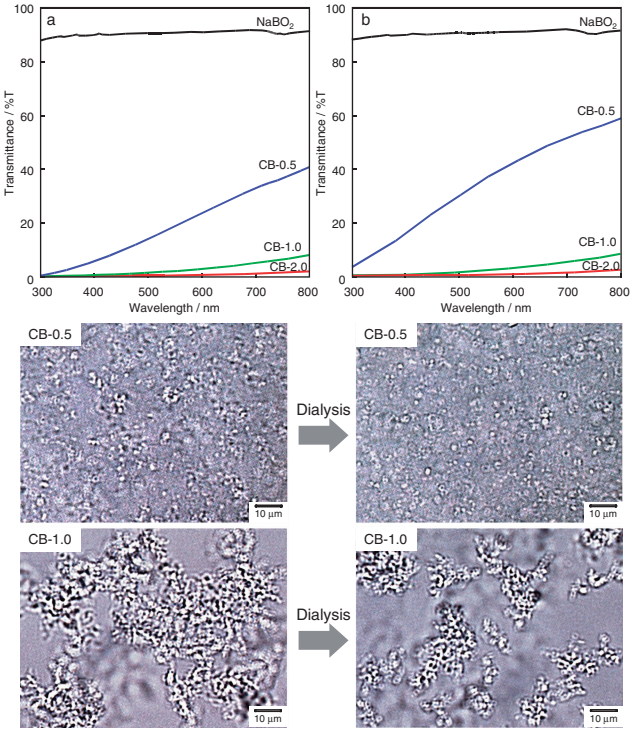

Figure 1. Optical transmittance for CB-*X* aqueous dispersion and $NaBO_2$. (a) Before and (b) after dialyzing. Boric acid addition: CB-0.5, 0.5 equiv for glucosamine unit of chitosan; CB-1.0, 1.0 equiv for glucosamine unit of chitosan; and CB-2.0, 2.0 equiv for glucosamine unit of chitosan. Microscope images: CB-0.5 and CB-1.0 aqueous dispersion.

The thermal stability of the chitosan–boric acid complexes for CB-0.5, CB-1.0, and CB-2.0 was investigated. Virtually no change in both the apparent degree of cloudiness and the transmittance after heat treatment at 80 °C for 12 h was observed. On the other hand, for comparison, chitosan particles that were ionically crosslinked using multivalent anions without covalent bonds were prepared,[11] and it was ascertained that their suspension became clear and finally dissolved in water by the same heat treatment. This is a distinct advantage of the borate crosslinking.

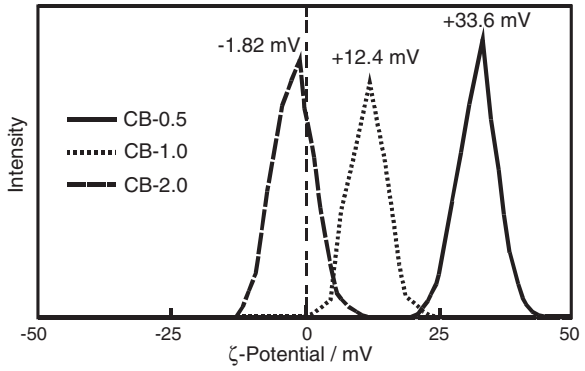

Figure 2. Zeta potential of aqueous chitosan–boric complex dispersion. Zeta potential were determined by laser Doppler method.

Figure 1 includes stereomicroscope photographs of the chitosan–boric acid complexes. In the case of CB-0.5, for which the degree of cloudiness is lowest, it is well-dispersed independent of the dialysis, and the average diameter was around 100 nm to 1 μm. On the other hand, in the case of the CB-1.0, agglutination between particles was seen prior to the dialysis, and the fact that dispersion is promoted by the dialysis was concluded. The average particle diameter after dialysis was around 1 μm. From the above, it was ascertained that the greater the amount of boric acid added, the greater the particle diameter and, in addition, the more likely agglutination becomes.

The particle diameter distribution of CB-0.5, CB-1.0, and CB-2.0 was evaluated using dynamic light scattering (DLS).[14] The tendency of the average particle diameter of all of the particles to be somewhat smaller with the dialysis was observed. It was found that the average particle diameter after dialysis is around 0.7 μm for CB-0.5, 2.6 μm for CB-1.0, and 1.9 μm for CB-2.0. However, it should be noted that because there is a tendency for CB-1.0 and CB-2.0 to agglutinate, it is presumed that the actual particle diameters are smaller.

Figure 2 shows the zeta potential of the chitosan particles after dialysis. The zeta potential increases in the order of CB-2.0 < CB-1.0 < CB-0.5 and it was observed that the zeta potential tended to shift toward the plus side the smaller the amount of boric acid added. In the stereomicroscope observations, the fact that the CB-0.5 dispersibility is higher than that of CB-2.0 and CB-1.0 was shown, but when inferred together with the zeta potential results, it is thought that free amino groups become most prevalent in CB-0.5. Because of this, the electrostatic repulsion between the particles themselves as well as hydration was promoted and the dispersibility has been increased. Incidentally, the amount of borate in the chitosan particles was ascertained by means of optical density employing azomethine H.[12] It was ascertained that the amount of contained borate increases in the order CB-0.5 (0.15 wt %[13]) < CB-1.0 (0.30 wt %[13]) < CB-2.0 (0.38 wt %[13]) in conformance with the preparation ratio.[14]

Using *E. coli* as a gram-negative bacterium and *A. niger* as a fungi, antimicrobial tests were performed. Commercially available chitosan powders (M_w = 70–100 kDa, deacetylation degree: 85 mol %, Hokkaido SODA Co., Ltd., Japan) were used for comparison. Figure 3 shows the results of pyrogenicity tests that

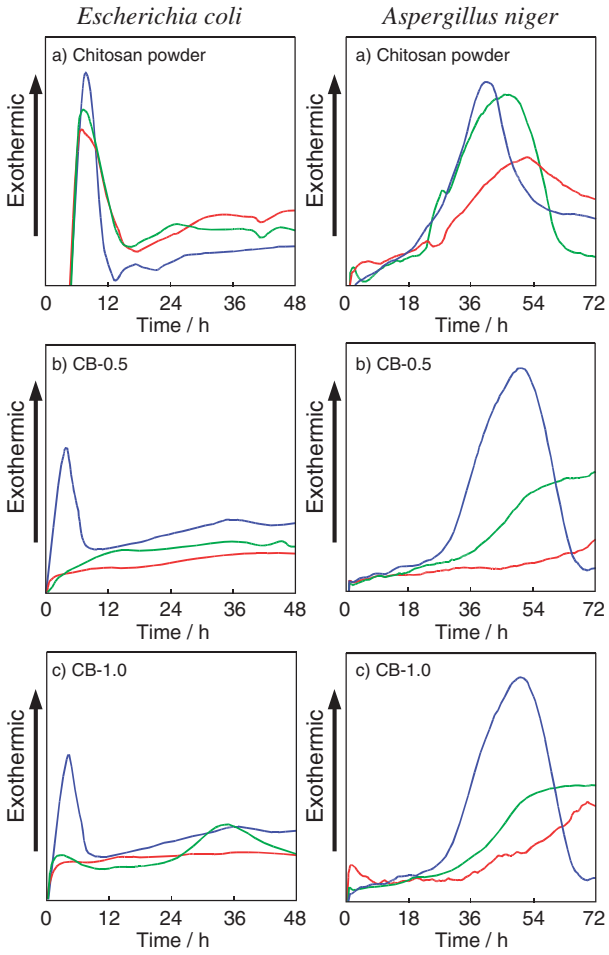

Figure 3. Antimicrobial activity for *E. coli* and *A. niger* of CB-0.5, CB-1.0, and chitosan powder. a) Chitosan powder (crushed products), b) CB-0.5, and c) CB-1.0. (—): 0 mg mL^{-1} for media, (—): 5.0 mg mL^{-1} for media, and (—): 10.0 mg mL^{-1} for media.

employed a microcalorimeter. The calorific value that is released with the cellular growth is proportional to a number of growing cellular. The change of calorific value with cellular growth is the differential form of growth curve and is provided as thermogram. Therefore, the exothermic peak in thermogram does not appear if the cellular growth is inhibited by antimicrobial materials. When the chitosan powders were added, there was no significant change in the pyrogenic pattern for both *E. coli* and *A. niger*: it is ascertained that there were almost no antimicrobial properties. In contrast to this, with the chitosan particles (CB-0.5 and CB-1.0) prepared in the present study, a distinct pyrogenic pattern change (a delay, reduction, and disappearance of the pyrogenic peak), in other words, a high degree of antimicrobial ability, was ascertained with regard to *E. coli* and *A. niger*.

In general, the positive charge of the amino groups of the chitosan is said to disturb the cell wall structure of the bacteria and fungi that are negatively charged. In other words, because the pK_a of the chitosan is 6.5, the chitosan has a sufficiently positive charge, and an acidic environment having a pH of 6 or less is necessary for ammonium ions to develop.[8] Since the results of the antimicrobial tests shown in Figure 3 are from

those carried out under conditions of a near neutral pH of 6.5, it is thought that antimicrobial activity was not obtained with the chitosan powders because of an insufficient amount of ammonium ions. In contrast to this, with the CB-*X* particles, more effective antimicrobial ability was exhibited despite the fact that the antimicrobial tests were under the same conditions. This difference is conjectured to be related to the fact that while on the one hand it is thought that perhaps the chitosan powders are almost nonporous and that the specific area for contact with the microbes is deficient, in the case of the CB-*X* particles, a reticular structure in which the specific area is large is formed (CB-0.5: 16.43 $m^2 g^{-1}$, CB-1.0: 8.52 $m^2 g^{-1}$, CB-2.0: 10.6 $m^2 g^{-1}$ and chitosan powder: 0.65 $m^2 g^{-1}$).[14] In other words, it is thought that because the sites for adsorption of the microbe cell walls become most prevalent, this leads to a high degree of antimicrobial activity.

In conclusion, we succeeded in the production of dispersible chitosan particles from several microns to submicron size by the making of a complex using an aqueous solution of chitosan lactate with an aqueous solution of sodium metaborate. The antibacterial and antifungal activities of the obtained complex particles were evaluated and compared with that of commercially available chitosan powders. As a result, it was ascertained that highly efficient antibacterial and antifungal activities at a neutral pH solution were exhibited only by our dispersible complex particles.

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13 The content of borate is ratio for weight of chitosan particles.

14 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

