Formation of Biocompatible Nanoparticles by Self-Assembly of Enzymatic Hydrolysates of Chitosan and Carboxymethyl Cellulose

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A simple preparation method for biocompatible nanoparticles in high concentration (0.5 wt %) by self-assembly of chitosan and carboxymethyl cellulose hydrolysates was developed. Chitosan and carboxymethyl cellulose were hydrolyzed beforehand with chitosanase and cellulase respectively to make fragments having lower molecular weights. Nanoparticles were spontaneously formed only by mixing the two hydrolysate solutions. The particle size distribution was relatively narrow, about 200 nm in mean size. The mean particle size decreased from 226 nm to 165 nm with decreasing molecular weight of chitosan hydrolysate from 9.5 to 6.8 kDa. The mixing ratio of chitosan and carboxymethyl cellulose hydrolysates also affected particle size. Changes in particle size are discussed in relation to a possible mechanism of polyionic complexation. The chitosan-carboxymethyl cellulose nanoparticles were stably suspended over 1 week even under low pH (pH 3.0), high ionic strength (NaCl 1M), or low temperature (4°C).

Key words: nanoparticle; chitosan; carboxymethyl cellulose; polyion complex; chitosanase

The Formation of nanoparticles by self-assembly is an energy-saving process, in contrast to the so-called “top-down” method. Self-assembling nanoparticles can be formed spontaneously under mild conditions. For carriers of drugs and functional food ingredients, these spontaneously formed nanoparticles are suitable to protect them from inactivation by intense shear stress, heat, and pH. Biodegradability and biocompatibility are also fundamental requirements that determine the possible therapeutic and surgical applications of nanoparticles as carriers.

Chitosan is a kind of natural polysaccharide, consisting of d-glucosamine, partially N-acetyl-d-glucosamine. It is a potentially useful pharmaceutical material owing to its good biocompatibility and low toxicity. For these reasons, it has been used as a material for carriers in delivery systems for biologically active substances. Previously chitosan microparticles were prepared using a cross-linking agent combined with an emulsification technique. Chitosan has also received attention as a material for nanoparticles for the last decade. Negatively charged compounds can be entrapped in chitosan-based nanoparticles by electrostatic interaction with positively charged chitosan. Chitosan-based nanoparticles containing DNA are studied for potential applications in gene delivery. Mucosal delivery of DNA has been investigated because chitosan has the special feature of adhering to mucosal surfaces. Physiological active components are also entrapped in chitosan-based nanoparticles, such as the anticancer drug doxorubicin and the cyclic polypeptide cyclosporin A.

Chitosan nanoparticles have been prepared using covalently crosslinking reagents such as glutaraldehyde, but most crosslinking reagents are highly cytotoxic, so they can impair biocompatibility. Nanoparticles can also be prepared by ionic complexation between cationic chitosan and specific polyanions, such as tripolyphosphate (TPP), DNA, and poly(acrylic acid), as candidates for a delivery carrier for biologically active substances.

In this study, we present a simple preparation method for nanoparticles in high concentration by self-assembly of chitosan and carboxymethyl cellulose (CMC) hydrolysates. CMC is also a natural polysaccharide as an acid derivative of cellulose. Nanoparticles composed of two kinds of natural polymers, viz., chitosan and CMC, must be biodegradable and biocompatible. Usually it is difficult to prepare a suspension of nano-size particles in high concentration by mixing intact chitosan and CMC solutions. We hypothesized that the molecular weights of polymers can affect the size of particles. Hence chitosan and CMC were hydrolyzed enzymatically to make their hydrolysates smaller in molecular weights.

Abbreviations: CMC, carboxymethyl cellulose; GPC, gel permeation chromatography

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The molecular weights that make possible the formation of nanoparticles were analyzed. The effects of pH, temperature, and ionic strength on the size and stability of chitosan-CMC nanoparticles were also examined.

Materials and Methods

Materials. Chitosan was obtained from Kimica (Tokyo). The molecular weight of the chitosan was about 400 kDa by viscometric measurement. The degree of deacetylation was 77% by infrared spectroscopy. Sodium carboxymethyl cellulose (CMC) was obtained from Tokyo Kasei Chemicals (Tokyo). The molecular formula of CMC was \([\text{C}_6\text{H}_{12}\text{O}_2(\text{OH})y^- (\text{OCH}_2\text{COONa})_x]_n\) (\(x = 2.42, \ y = 0.58, \ n = \) about 1,050). The degree of substitution of CMC was determined by ashing and titration. Other chemicals were of reagent grade.

Enzymes. Chitosanase (from *Bacillus pumilus* BN-262, 47,000 units/mg of powder) was supplied by Meiji Seika Kaisha (Tokyo). Chitosanase was immobilized on the surface of Sepharose 6B (Pharmacia, Uppsala, Sweden) as support material by the multipoint attachment method. Immobilization of chitosanase was performed by following the procedure described previously. The activity of immobilized chitosanase was 0.05 units/g of support. Cellulase (from *Trichoderma viride*, 96.8 units/mg of powder) was obtained from Amano Enzyme (Nagoya, Japan).

Enzymatic hydrolysis of chitosan and CMC. Chitosan powder (0.5 g) was added to acetic acid (1 ml) and then dissolved in water (99 ml) with stirring. The chitosan solution was adjusted to pH 5.6 with an NaOH solution. For hydrolysis of chitosan, immobilized chitosanase (3 g of support) was added to 100 ml of the chitosan solution. The reaction mixture was incubated at 45 °C for 0.5–7 h with stirring. The hydrolysis reaction was stopped by separating immobilized chitosanase from the reaction mixture.

CMC (0.5 g) was dissolved in water (100 ml) and then adjusted to pH 5.6 with HCl. The CMC solution was hydrolyzed with cellulase (5 mg) at 45 °C for 3 h. Hydrolysis of CMC was stopped by thermal inactivation of cellulase.

Molecular weight distributions of chitosan and CMC hydrolysates. The molecular weight distributions of chitosan and CMC hydrolysates were analyzed using a gel permeation chromatograph (GPC). Each of the chitosan and CMC hydrolysates (0.1 ml) was applied on a column (TSKgel G3000PWXL, 175 mm × 30 cm, Tosoh, Tokyo) and then eluted with pure water at a flow rate of 1 ml/min at 20 °C using HPLC equipped with an RI detector. A kit of pullulan preparations (Shodex P-82, Showa Denko, Tokyo) was used as molecular weight markers.

Measurement of particle size. The size of the nanoparticles was measured by dynamic light scattering. The diffusion coefficient of nanoparticles was estimated by the auto-correlation function measured at 90° scattering angle with the dynamic light scattering instrument DLS-7000 (Otsuka Electronics, Osaka, Japan) with Ar laser (488 nm). The hydrodynamic diameter was calculated from the diffusion coefficient using the Stokes–Einstein equation, and then used as an index of mean particle size.

Results and Discussion

Preparation of chitosan-CMC nanoparticles

Preparation of nano-size particles in high concentration using intact chitosan and CMC solutions was difficult because large gel-particles of drop-size formed with mixing of the two solutions. When chitosan and CMC were hydrolyzed to make fragments having smaller molecular weights, nano-size particles were formed easily only by mixing their hydrolysate solutions. We developed the following process: Chitosan (0.5 wt %) was hydrolyzed with immobilized chitosanase. On the other hand, CMC (0.5 wt %) was also hydrolyzed with cellulase for 3 h. The same volumes of the chitosan and CMC hydrolysates were mixed at pH 5.6 with mild stirring, and then the mixture became an opalescent suspension because nano-size particles formed spontaneously. The total concentration of chitosan and CMC in the suspension was 0.5 wt %. Biocompatible nanoparticles in higher concentration can be prepared by this simple procedure.

A schematic representation of the formation of chitosan-CMC nanoparticles is shown in Fig. 1. The pKa values of the chitosan amino group and the CMC carboxyl group are 6.3 and 3.0 respectively. Since the pH condition at mixing was 5.6, both functional groups were charged. When CMC hydrolysate was mixed with chitosan hydrolysate, inter-molecular linkages occurred between negatively charged carboxyl groups and positively charged amino groups. These ionic linkages caused the self-assembly of chitosan and CMC polymers, and then nanoparticles formed spontaneously via this polyelectrolyte complexation.

Effect of the molecular weights of hydrolysates on particle size

In order to examine the effect of the enzymatic fragmentation of chitosan, nanoparticles were prepared using chitosan hydrolysates prepared with different hydrolysis reaction times. The effect of hydrolysis reaction time on the mean size of chitosan-CMC nanoparticles is shown in Table 1. When the hydrolysis reaction time was 0.5 h, the size was 226 nm. Under this condition, the formation of large precipitates of micro-size was observed, but when chitosan was hydrolyzed more than 2 h, no precipitation formed. With increasing reaction time, the mean particle size
The molecular weight of CMC hydrolysate was 11 kDa. The number of carboxyl groups in one molecule of CMC hydrolysate was 31. Chitosan (0.5 wt%) and CMC (0.5 wt%) hydrolysate solutions were mixed.

Table 1. Effect of Molecular Weight of Chitosan Hydrolysate on Particle Size

<table>
<thead>
<tr>
<th>Hydrolysis reaction time (h)</th>
<th>Molecular weight (kDa)</th>
<th>Number of amino groups in one chitosan hydrolysate (—)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>400</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>9.5</td>
<td>47</td>
<td>226</td>
</tr>
<tr>
<td>2.0</td>
<td>8.1</td>
<td>40</td>
<td>213</td>
</tr>
<tr>
<td>4.0</td>
<td>7.3</td>
<td>36</td>
<td>183</td>
</tr>
<tr>
<td>6.0</td>
<td>6.8</td>
<td>33</td>
<td>165</td>
</tr>
</tbody>
</table>

The number of amino groups in one molecule of CMC hydrolysate was also estimated to be 31 by reference to their molecular weights and degrees of substitution. Under the condition of 6 h of hydrolysis, the number of amino groups in one chitosan hydrolysate, 33, was comparable to that of the carboxyl groups in one CMC hydrolysate, 31. In this case, nanoparticles are formed preferentially by side-by-side ionic linkages (schematically shown in Fig. 1) between chitosan and CMC hydrolysates. This promotes the formation of smaller particles. On the other hand, when the number of amino groups was rather larger compared to the number of carboxyl groups under the shorter hydrolysis reaction condition in Table 1, a chitosan hydrolysate molecule linked to a larger number of CMC hydrolysate molecules by further end-to-end and/or
random ionic linkages (schematically shown in Fig. 1). This type of polyionic complexation allows the formation of larger particles.

**Size distributions of chitosan-CMC nanoparticles**

The cumulative size distribution of nanoparticles is shown in Fig. 3. It was obtained by a second-order cumulant analysis of the dynamic light scattering data. The hydrolysis reaction time of chitosan was 4 h. The mean particle size was 183 nm. The size distribution was narrow considering the simple preparation method mixing of the two hydrolysates solutions. This was perhaps caused by the narrow molecular weight distribution of chitosan hydrolysate, as shown in Fig. 2.

**Effect of the mixing ratio of chitosan and CMC hydrolysates**

Mean particle size was affected by the mixing ratio of chitosan and CMC hydrolysates, as summarized in Table 2. From the practical viewpoint, control of particle size is possible by changing the mixing ratio. The molar ratio of total amino to total carboxyl groups in the mixed suspension, *i.e.*, the theoretical charge ratio, is also shown in Table 2. When the molar ratio of chitosan:CMC hydrolysates was 60:40 (total amino: carboxyl groups = 64:36), the smallest particle was formed under three different conditions. The appropriate mixing ratio caused the side-by-side ionic linkages prior to the end-to-end and/or random linkages between chitosan and CMC hydrolysates, and then allowed the formation of smaller particles. Otherwise, the excess of one type of hydrolysate over the other would induce the multiple interaction of one hydrolysate molecule with many other molecules. This type of complexation allowed the formation of larger particles.

**Effects of pH, temperature, and ionic strength on particle size and stability**

The effects of pH, storage temperature, and ionic strength on the size and stability of particles were examined. Particles were prepared at pH 5.6 and 25 °C, and then were left standing under the same conditions (Fig. 4a). The mean particle size was 172 nm after 7 d, while the initial value was 183 nm. It changed little and no precipitation was observed during storage. The particles were stably suspended, keeping their size.

The pH condition was changed from 5.6 to 3.0 by the addition of HClaq immediately after preparation, and

![Fig. 3. Cumulative Size Distribution of Chitosan-CMC Nanoparticles.](image1)

The hydrolysis reaction times of chitosan and CMC were 4 h and 3 h, respectively. Nanoparticles were made by mixing chitosan and CMC hydrolysates solutions at pH 5.6. The mean size was 183 nm.

![Fig. 4. Effects of pH, Temperature, and Ionic Strength on Mean Particle Size and Stability.](image2)

**Table 2. Change in Particle Size with Mixing Ratio of Chitosan and CMC Hydrolysates**

<table>
<thead>
<tr>
<th>Molar ratio Chitosan:CMC hydrolysates</th>
<th>Amino:Carboxyl groups</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43:57</td>
<td>47:53</td>
<td>218</td>
</tr>
<tr>
<td>60:40</td>
<td>64:36</td>
<td>183</td>
</tr>
<tr>
<td>75:25</td>
<td>78:22</td>
<td>233</td>
</tr>
</tbody>
</table>

The molecular weights of chitosan and CMC hydrolysate were 7.3 kDa and 11 kDa, respectively. The number of amino groups in one molecule of chitosan hydrolysate was 36. The number of carboxyl groups in one molecule of CMC hydrolysate was 31. The total concentration of chitosan and CMC was 0.5 wt%.
then the suspension was left standing at 25 °C (Fig. 4b). The initial size immediately after the addition of HCl\textsubscript{aq} was 206 nm, and then it was 182 nm after 7 d. This small change in size was probably caused by the rearrangement of ionic linkages from the initial state of complexation toward a stable state.\textsuperscript{31} The size became slightly larger than without HCl\textsubscript{aq} addition, as shown in Fig. 4a. The charge density of CMC fragments on at pH 3.0 is low, and consequently their loose electrostatic linkages with chitosan fragments would cause swelling of the particles.

The ionic strength was changed to 1 M NaCl on pH 3.0 (Fig. 4c). In spite of this high ionic strength, the particles were stably suspended during 7 d of storage at 25 °C. The mean size of the particles after 7 d was 200 nm. It became larger than that without NaCl addition at pH 3.0, as shown in Fig. 4b. The ionic linkages between chitosan and CMC hydrolysates were weakened by the charge screening effect, and then the particles became loose and swollen.

The particle suspension was left standing at 4 °C immediately after the pH was changed from 5.6 to 3.0 (Fig. 4d). The difference in conditions between Fig. 4b and d was only storage temperature. The initial mean size was 204 nm, and then it became 186 nm after 7 d. There was no big change in particle size, as compared with Fig. 4b. Size and stability were not affected by low temperature.

A series of experiments proved the high stability of particle suspension under low pH (pH 3.0), high ionic strength (NaCl 1 M), or low temperature (4 °C) conditions in spite of the simplicity of the preparation method.

In conclusion, biocompatible nanoparticles in high concentration (0.5 wt %) can be prepared by self-assembly of hydrolysates of chitosan and CMC. Chitosan and CMC were hydrolyzed beforehand with chitosanase and cellulase respectively. Nanoparticles formed spontaneously only with mixing of the two hydrolysate solutions at pH 5.6 via polyanion complexation. Their size distribution was relatively narrow, about 200 nm in mean size. Mean particle size decreased from 226 nm to 165 nm with decreasing molecular weight of chitosan hydrolysate from 9.5 kDa to 6.8 kDa. The mixing ratio of chitosan and CMC hydrolysates also affected particle size. Changes in particle size are discussed in relation to a possible mechanism of polyionic complexation. It was found that the particle suspensions were stable over 1 week even at low pH (pH 3.0), high ionic strength (NaCl 1 M), or low temperature (4 °C) conditions.

Using this type of chitosan-CMC nanoparticle, negatively charged DNA fragments and amphotropic peptide and protein were entrapped efficiently.\textsuperscript{32} Positively charged compounds were also entrapped by mixing them with CMC hydrolysate, followed by the addition of chitosan hydrolysate to form nanoparticles.\textsuperscript{32} This simple method for chitosan-CMC nanoparticles should be useful for the preparation of carriers of drugs and functional ingredients in the pharmaceutical and food industries.

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