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Autoclaving Achieves pH-Neutralization, Hydrogelation, and Sterilization of Chitosan Hydrogels in One Step

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Abstract: Conventionally, chitosan hydrogels are acidic and contain toxic chemicals because chitosan is soluble only in acidic solvents and requires toxic additives such as chemical crosslinkers and polymerization agents to fabricate chitosan hydrogels. These properties prevent chitosan hydrogels from being used for medical applications. In this study, chitosan hydrogels were prepared by a simple and versatile process using urea hydrolysis by autoclaving (steam sterilization, 121 °C, 20 min). When autoclaved, urea hydrolyzes in an acidic chitosan aqueous solution, and ammonia is produced, which increases the pH of the solution, and chitosan becomes insoluble, leading to the formation of a chitosan hydrogel. The pH and osmotic concentration of chitosan hydrogels could be adjusted to be suitable for physiological conditions (pH: 7.0–7.5, and osmotic concentration: 276–329 mOsm/L) by changing the amount of urea added to chitosan solutions (chitosan: 2.5% (*w*/*v*), urea: 0.75–1.0% (*w*/*v*), pH: 5.5). The hydrogels had extremely low cytotoxicity without the washing process. In addition, not only pure chitosan hydrogels, but also chitosan derivative hydrogels were prepared using this method. The autoclaving technique for preparing low-toxic and wash-free sterilized chitosan hydrogels in a single step is practical for medical applications.

Keywords: chitosan; hydrogel; urea; autoclaving; one-step preparation; wash-free

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1. Introduction

Chitosan is a cationic polysaccharide which is composed of D-glucosamine and N-acetylglucosamine in random repeats [1]. The polymer exists in the cell walls of adhesive fungi in natural resources. Industrially, chitosan is obtained by deacetylation of chitin, a natural polysaccharide found in the exoskeletons of crustaceans such as shrimp and crabs through the hot alkaline hydrolysis of chitin. Among these polymers, those characterized by a degree of deacetylation exceeding 50% are termed chitosan, while those with a degree of deacetylation below 50% are termed chitin. Chitosan is known for its favorable biomedical properties such as biocompatibility, biodegradability, antimicrobial activity, hemostatic properties, and wound healing properties [1]. The polymer has been formed into various forms such as fibers [2], fine particles [3], sponges [4], and hydrogels [5] used as biomaterials. Among those materials, hydrogels are the most suitable for medical applications because they resemble living tissue, contain a large amount of water, are highly flexible, and have high material permeability. Therefore, chitosan hydrogels have been used as wound dressing [6,7], carriers of drugs [8,9], and scaffolds for nerve [10], cartilage [11], bone regeneration [12], and so forth.

However, conventionally, chitosan hydrogels are acidic because chitosan is soluble only in acidic solvents [1]. To improve the solubility of chitosan, various chitosan derivatives such as carboxymethyl chitosan, quarternized chitosan, and succinyl chitosan have been synthesized that dissolve in pH-neutral water [13–15]. However, they require toxic chemical crosslinkers and polymerization agents for gelation [16,17]. To solve the problem, chitosan derivatives that physically cross-link without using toxic chemical cross-linkers have

been developed. For example, chitosan methacrylate formed a hydrogel when its neutral aqueous solution was exposed to UV light [18]. Chitosan–gallic-acid conjugate has been reported to be enzymatically gellable by horseradish peroxidase [19]. These hydrogels do not contain toxic chemicals, and are therefore suitable for medical applications.

We previously reported that chitosan–gluconic-acid conjugate (CG) was soluble in a pH-neutral aqueous solution [20,21], and the solution formed a physically crosslinked hydrogel by autoclaving [22], which is steam sterilization at high temperatures and high pressure (121 °C, 2 atm), without toxic additives. The CG hydrogel has good biological properties derived from chitosan for wound healing. The technique in which the CG solution formed hydrogels and was sterilized simultaneously was practical. In the previous research, CG was first dissolved in acidic water, and then neutralized by adding alkali. The pH-neutral CG solution was autoclaved to obtain a CG hydrogel. Thus, in the previous method, two steps (pH neutralization and autoclaving) were required to obtain a hydrogel from an acidic CG solution. To reduce the number of processes to produce hydrogels is practical. In addition, while conventional chitosan hydrogels consist of chitosan derivatives, it would be desirable for hydrogels made from pure chitosan to be produced.

In this study, we focused on the hydrolysis reaction of urea at high temperatures [23,24]. Hydrolysis of urea, generating ammonia, and an increase in the pH of acidic chitosan aqueous solution would be expected to insolubilize the chitosan and form a physically crosslinked hydrogel in one step.

The purpose of this study was to propose a one-step preparation technique for low-toxic, wash-free, and sterilized chitosan hydrogels. We successfully prepared chitosan hydrogels suitable for physiological pH, osmotic concentration, and acceptable concentration of ammonia. Furthermore, this approach would be versatile because it could produce hydrogels composed of chitosan derivatives as well as pure chitosan. The technology was considered very practical for the application of chitosan hydrogels in the medical field.

2. Materials and Methods

2.1. Materials

Chitosan (degree of deacetylation: 83%) was purchased from KIMICA Corporation (Tokyo, Japan). Urea was purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). CG was synthesized by condensation of chitosan and gluconic acid via carbodiimide chemistry according to our previously reported method [20]. Triton X-100 was purchased from MP Biomedicals (Santa Ana, CA, USA). WST-8 [2-(2-methoxy-4-nitrophenyl)-2H-tetrazolium monosodium salt] kit was purchased from Dojindo Laboratories. Mouse fibroblast L929 (RCB1451) cells were purchased from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in a minimum essential medium (MEM) containing 10% fetal bovine serum.

2.2. Preparation of Chitosan Hydrogels

Chitosan and CG (gluconic acid content: 13%) were dissolved in a dilute hydrochloric acid at a concentration of 2.5% (*w*/*v*) (Table 1). Urea was dissolved in the acidic chitosan solutions at a concentration of 0.0–1.5% (*w*/*v*). The pH of the chitosan solution was adjusted to 5.5. The pH of chitosan solutions and hydrogels was measured by soaking the sensor in the solution and piercing the hydrogel with a piercing pH meter (SPH70, AS ONE Corporation, Osaka, Japan), respectively. The chitosan solution containing urea was autoclaved (121 °C, 20–240 min).

Table 1. The conditions for the preparation of chitosan hydrogels.

	Concentration of Chitosan [% (*w*/*v*)]	Concentration of Urea [% (*w*/*v*)]	pH (Pre-Autoclaving)	pH (Post-Autoclaving)	Autoclaving Time [min]	Gelation
a	2.5	0.0	5.5 ± 0.0	5.5 ± 0.0	20	–
b	2.5	0.25	5.5 ± 0.0	6.0 ± 0.0	20	–

Table 1. *Cont.*

	Concentration of Chitosan [% (*w*/*v*)]	Concentration of Urea [% (*w*/*v*)]	pH (Pre-Autoclaving)	pH (Post-Autoclaving)	Autoclaving Time [min]	Gelation
c	2.5	0.5	5.5 ± 0.0	6.5 ± 0.0	20	−
d	2.5	0.75	5.5 ± 0.0	7.0 ± 0.0	20	+
e	2.5	1.0	5.5 ± 0.0	7.5 ± 0.1	20	+
f	2.5	1.25	5.5 ± 0.0	7.7 ± 0.0	20	+
g	2.5	1.5	5.5 ± 0.0	8.1 ± 0.0	20	+
h	2.5	1.0	5.5 ± 0.0	8.3 ± 0.0	60	+
i	2.5	1.0	5.5 ± 0.0	8.4 ± 0.1	120	+

+: Gelled. −: Did not gel.

2.3. The Rate of Hydrolysis of Urea and Osmotic Concentration

The urea hydrolysis reaction for gelation of chitosan solutions is described by the following equation:

$$(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2 \quad (1)$$

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^- \quad (2)$$

$$Chi{-}NH_3^+ + OH^- \rightarrow Chi{-}NH_2 + H_2O \quad (3)$$

where $Chi{-}NH_3^+$ is the protonated amino group of chitosan and $Chi{-}NH_2$ is the unchanged amino group of chitosan. The hydrolysis rate of urea was calculated from the amount of OH^- produced in these reactions by the following equation:

$$\text{Hydrolysis rate of urea } [\%] = \frac{\text{The amount of hydrolyzed urea calculated from pH change [mol]}}{\text{The amount of urea dissolved in chitosan solution before autoclaving [mol]}} \times 100 \quad (4)$$

where the pH change was the difference between the pH of a chitosan solution before and after autoclaving. The amount of OH^- produced by the urea hydrolysis reaction is the sum of the amount of OH^- that contributed to the increase in pH of a chitosan aqueous solution and the amount of OH^- that contributed to the deprotonation of amino groups of chitosan. The calculation method is shown in detail in the Appendix A.

These calculations gave the concentration of materials (urea, NH_4^+, NH_3, Cl^-) in the chitosan hydrogel after autoclaving. The osmotic concentration of chitosan hydrogels was calculated by the following equation:

$$\text{Osmotic concentration } [\%] = \text{the total concentration of urea, } NH_4^+, \text{ } NH_3, \text{ and } Cl^- \text{in the chitosan hydrogel} \quad (5)$$

2.4. Evaluation of Swelling

Chitosan hydrogels were prepared by autoclaving (121 °C, 20 min) 2.5% (*w*/*v*) chitosan solutions with different concentrations of urea (0.75–1.5% (*w*/*v*)) in a cylindrical glass container (15 mm in diameter, 8 mm in height). The hydrogels were immersed in phosphate-buffered saline without calcium and magnesium ions (pH = 7.4) at 37 °C. The wet weight of the hydrogels was measured every day. The degree of swelling was determined by the following equation:

$$\text{The degree of swelling } [\%] = \frac{\text{Hydrogel wet weight at day n [g]}}{\text{Hydrogel wet weight at day 0 [g]}} \times 100 \quad (6)$$

2.5. Compression Test

Chitosan hydrogels were prepared by autoclaving (121 °C, 20 min) 2.5% (*w*/*v*) chitosan solution with different concentrations of urea (0.75–1.5% (*w*/*v*)) in a cylindrical glass container (15 mm in diameter, 8 mm in height). The hydrogels were compressed as they were prepared in glass vials. The compression test was performed using a compact table-top universal tester (EZ-SX, SHIMADZU, Kyoto, Japan) at a rate of 5 mm/min.

2.6. Evaluation of Cytotoxicity

In this study, the cytotoxicity of chitosan hydrogel prepared under condition e (Table 1, pH: 7.5, osmotic pressure: 330 mOsm/L) was investigated according to previous reported methods [25,26]. L929 fibroblast cells were seeded on a 24-well culture plate at a density of 6.0×10^4 cells/well (0.4 mL medium/well) and precultured for 24 h. Chitosan hydrogels were freeze-dried and powdered. The chitosan hydrogel powder was sterilized with immersion in 70% ethanol for 10 min, and then the ethanol aqueous solution was completely evaporated with vacuum drying. The powder (2.5 mg) was immersed in 0.5 mL growth medium for 24 h at 37 °C to extract water-soluble components of chitosan hydrogels. After the growth medium was removed from wells in which cells were precultured, the conditioned medium (0.4 mL) as prepared was added to each well, and cells were cultured for 24 h. As a positive and negative control, fresh growth medium with 0.1% (*w*/*v*) Triton X-100 (surfactant), and growth medium without additives were used, respectively. After removing the medium from the wells, cells were rinsed with fresh growth medium. Subsequently, each cell was cultured in 0.4 mL of growth medium containing 10% (*w*/*v*) WST-8 reagent for 1 h. The absorbance of the medium at a wavelength of 450 nm was measured using a spectrophotometer (Infinite 200 PRO MPlex, Tecan, Männedorf, Switzerland).

3. Results and Discussion

3.1. Preparation of Chitosan Hydrogels

Generally, the solubility of chitosan is known to be pH dependent. Chitosan is insoluble when the pH is higher than 6.5 due to its rigid crystalline structure, while in acidic solutions lower than pH 6.5, the protonation of the amino groups causes electrostatic repulsion between polymers, resulting in dissolution. In this research, urea was used to increase the pH of a chitosan aqueous solution to form a hydrogel. When urea is heated in an aqueous solution, it hydrolyzes to ammonia, increasing the pH. The increase in pH was expected to cause chitosan to become insoluble, forming a hydrogel.

In this study, chitosan hydrogels were prepared under several conditions, shown in Table 1, to investigate (i) the effect of the amount of urea, and (ii) autoclaving time on hydrogelation of a chitosan solution and characteristics of chitosan hydrogels. Figure 1 shows the appearance of chitosan hydrogels prepared under these conditions. Under conditions a–g, the effect of the amount of urea added to the chitosan aqueous solution on hydrogel formation was investigated. Chitosan aqueous solutions formed hydrogels when the concentration of urea was more than 0.75% (*w*/*v*), while chitosan aqueous solutions did not form hydrogels when the urea concentration was less than 0.5% (*w*/*v*) (Figure 1a–g). This is due to the pH of the solution after autoclaving, since chitosan is insoluble when the pH is more than 6.5, which is the pKa of chitosan. In conditions f and g, the chitosan hydrogels got clouded after autoclaving (Figure 1f,g). We considered the cloudiness of the chitosan hydrogels to be attributed to the thickness of the hydrogel skeleton due to the aggregation of chitosan in the hydrogel. However, that could not be observed by optical or electron microscopy because of the difficulty of drying the hydrogels without deformation. The different appearance of these hydrogels suggests that the properties of these hydrogels are different. Therefore, the physical properties of these hydrogels were investigated later. Then, in conditions e, h, i, the effect of autoclaving time on the formation of the chitosan hydrogels was investigated. As a result, it was found that the pH of the chitosan hydrogels increased as the autoclaving time was extended. This indicates that the urea hydrolysis reaction occurred gradually, and that the pH of the chitosan hydrogels can also be controlled by extending the autoclaving time.

Usually, to neutralize an acidic chitosan aqueous solution, an alkali solution is gradually added dropwise to it. However, in the method, the pH of a chitosan solution partially rises to much higher, and precipitates chitosan. This results in a non-uniform structure of the hydrogel, making it difficult to produce a hydrogel of stable quality. On the other hand, in this method, the urea in the acidic chitosan solution hydrolyzed, which uniformly raises

the pH of the entire solution, resulting in uniform gelation of a chitosan aqueous solution, very easily.

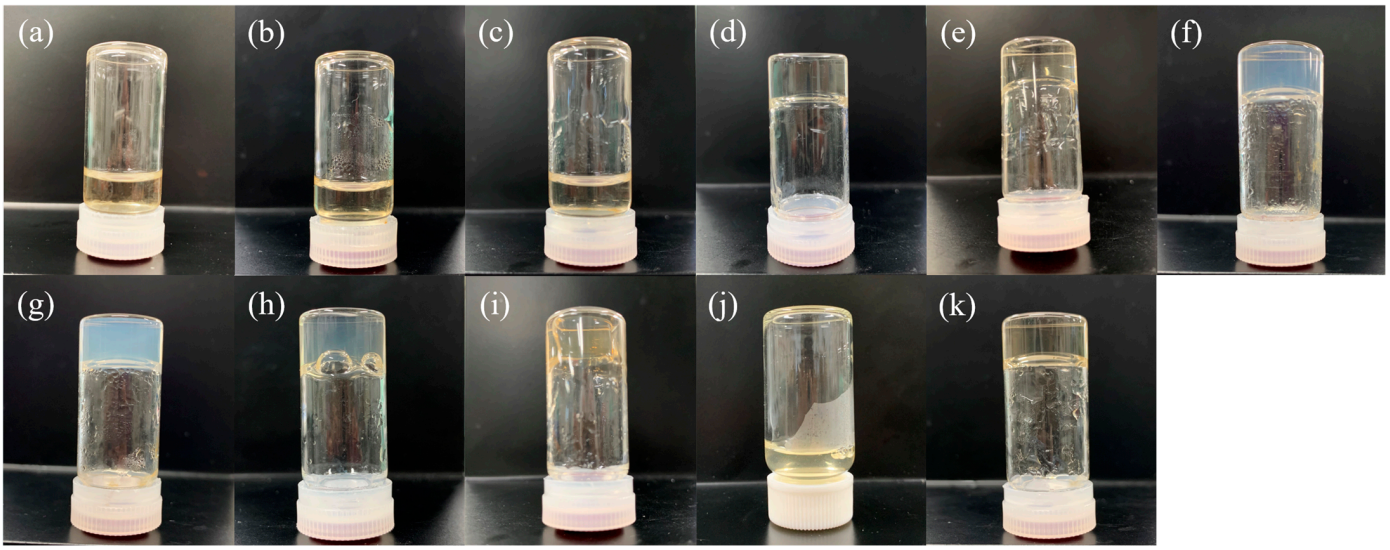


Figure 1. (**a**–**i**) The chitosan hydrogels prepared under conditions a–i in Table 1, and (**j**) chitosan aqueous solution heated at 100 °C, 6 h, and (**k**) CG hydrogel prepared by autoclaving (121 °C, 20 min).

Figure 2 shows the hydrolysis rate of urea and the pH of the chitosan hydrogels prepared by various conditions. The pH of the chitosan hydrogels increased as the amount of urea in the chitosan solution increased (Figure 2a). The hydrolysis rate of urea increased in a concentration of urea less than 0.75% (*w*/*v*) with increase in the concentration, but decreased in higher concentration of urea. The hydrolysis rate of urea and the pH of chitosan hydrogels increased as autoclaving time was extended (Figure 2b). The results indicate that the hydrolysis rate of urea is slow, and furthermore, the pH of chitosan hydrogels can be controlled by changing autoclaving time.

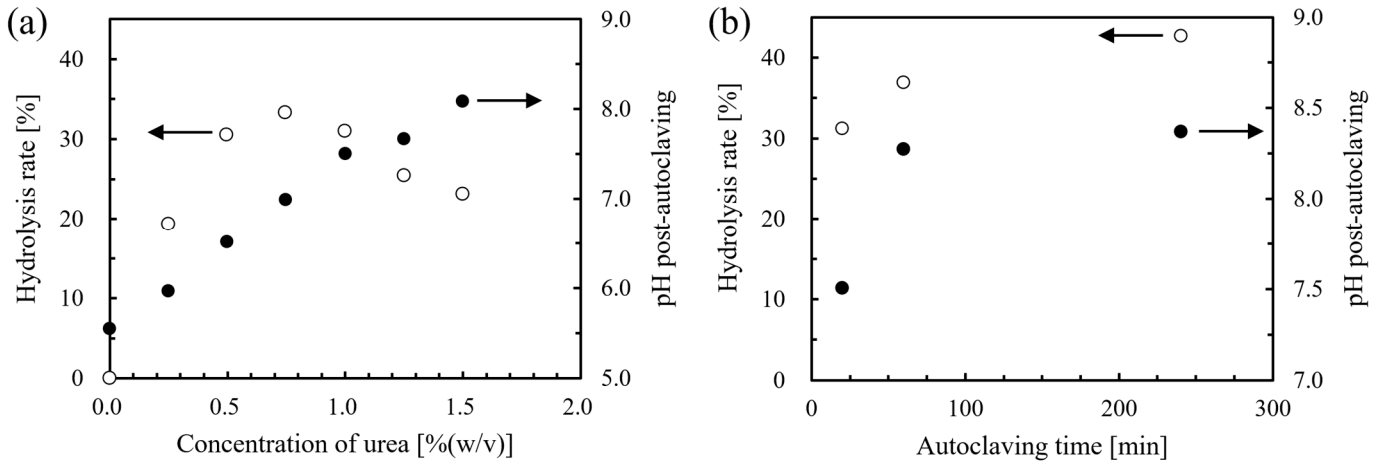


Figure 2. The pH and the hydrolysis rate of urea of the chitosan hydrogels prepared under various conditions. (**a**) The influence of amount of urea (0.0–1.5% (*w*/*v*)), and (**b**) the influence of autoclaving time (20–240 min).

In this research, autoclave sterilization (121 °C, 2 atm) was used mainly to hydrolyze urea. It was also investigated whether chitosan hydrogels could be produced with heating below 100 °C. However, even after heating at 100 °C for 6 h, the pH of the chitosan aqueous solution hardly increased, and the solution did not form hydrogels (Figure 1j). This might be because the reaction rate of urea hydrolysis is very slow at temperatures below 100 °C, and

heating at 100 °C for several hours is not sufficient to raise the pH of the chitosan aqueous solution. Autoclave sterilization is therefore an essential operation in the preparation of chitosan hydrogels using the urea hydrolysis reaction.

Furthermore, we investigated the possibility of preparing hydrogels with chitosan derivatives as well as pure chitosan as a structure by the urea method. Herein, chitosan-gluconic acid conjugate (CG, chitosan chemically modified with gluconic acid on the amino groups) was used as a chitosan derivative [20–22]. The CG hydrogels were also successfully prepared using the urea method (Figure 1k). This shows the potential versatility of the technique.

3.2. Swelling Behavior

The swelling behavior is important for hydrogels as medical materials. Thus, we investigated the swelling behavior of chitosan hydrogels prepared using autoclaving. Figure 3 shows the degree of swelling of chitosan hydrogels prepared using autoclaving chitosan aqueous solution with different concentrations of urea (0.75–1.5% (*w*/*v*)). Initially, after immersion, chitosan hydrogels prepared from chitosan aqueous solutions containing 1.25 and 1.5% (*w*/*v*) urea shrank, while those prepared from chitosan aqueous solutions containing 0.75 and 1.0% (*w*/*v*) urea swelled. This property may be attributed to the structure of chitosan hydrogels. The thicker the hydrogel skeleton (1.25, 1.5% (*w*/*v*) of urea), the smaller the surface area and therefore the smaller the water holding capacity. Conversely, the thinner the skeleton of the hydrogel (0.75, 1.0% (*w*/*v*) of urea), the larger the surface area, and therefore the more water the hydrogel holds. Since chitosan is normally insoluble in water, the degree of swelling of any chitosan hydrogels hardly changed at all from day 3 to day 7. Generally, it is known that the mechanical properties of hydrogels strongly depend on the degree of swelling. The hydrogels are useful because their mechanical properties can be controlled simply by changing the concentrations of urea in chitosan aqueous solutions. In addition, the controllability of swelling behavior of the hydrogels might be applied to drug release. Hydrogels with greater swelling are considered to release drugs faster, while those with less swelling are considered to release drugs slowly.

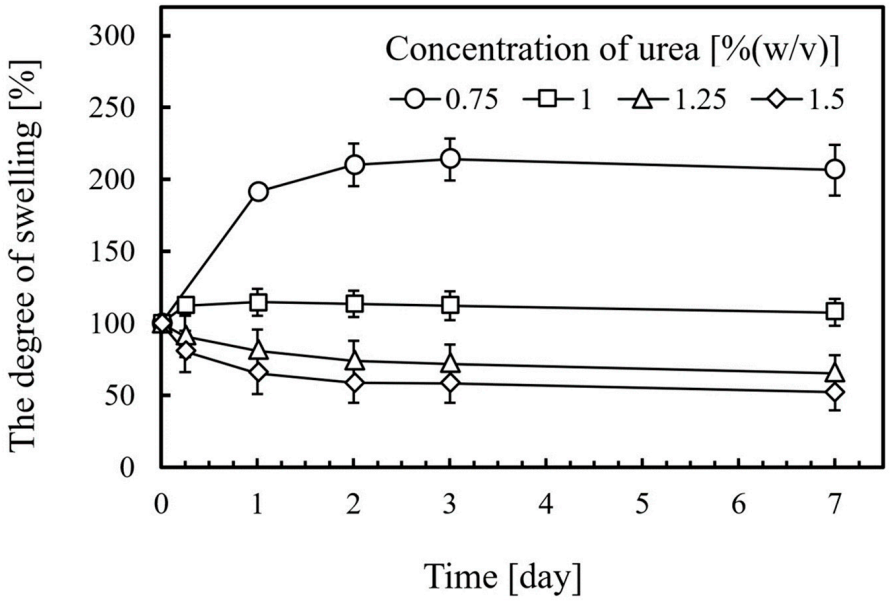


Figure 3. The degree of swelling of chitosan hydrogels prepared by autoclaving chitosan aqueous solutions with different concentrations of urea (0.75–1.5% (*w*/*v*): condition d–g) (n = 3).

3.3. Mechanical Properties

The mechanical properties of hydrogels are very important for their use as biomaterials. Therefore, compression tests were performed to investigate the mechanical properties of chitosan hydrogels prepared by autoclaving chitosan aqueous solutions with different concentrations of urea (0.75–1.5% (*w*/*v*)). Figure 4 shows the typical strain-compressive strength curve of the chitosan hydrogels and their breaking strength. The strength of the hydrogels varied greatly depending on the concentrations of urea. Chitosan hydrogels prepared from chitosan aqueous solutions containing 0.75, 1.0, 1.25, and 1.5% (*w*/*v*) had a breaking strength of 1.46, 1.96, 13.1, and 26.9 N (average), respectively. These results indicate that the higher the concentrations of urea in the chitosan solution before autoclaving, the higher their strength and resistance to strain. The mechanical properties of the hydrogels could be controlled by changing only the urea concentration of the chitosan aqueous solution to suit the application. Hydrogels with high-strain (0.75 and 1.0% (*w*/*v*) of urea) could conform to the complex shapes of tissues when implanted in the body, making them suitable for use as wound dressings. On the other hand, hydrogels with high-strength (1.25 and 1.5% (*w*/*v*) of urea) could be used as tissue engineering scaffolds for tissues under a constant load such as cartilage and bone.

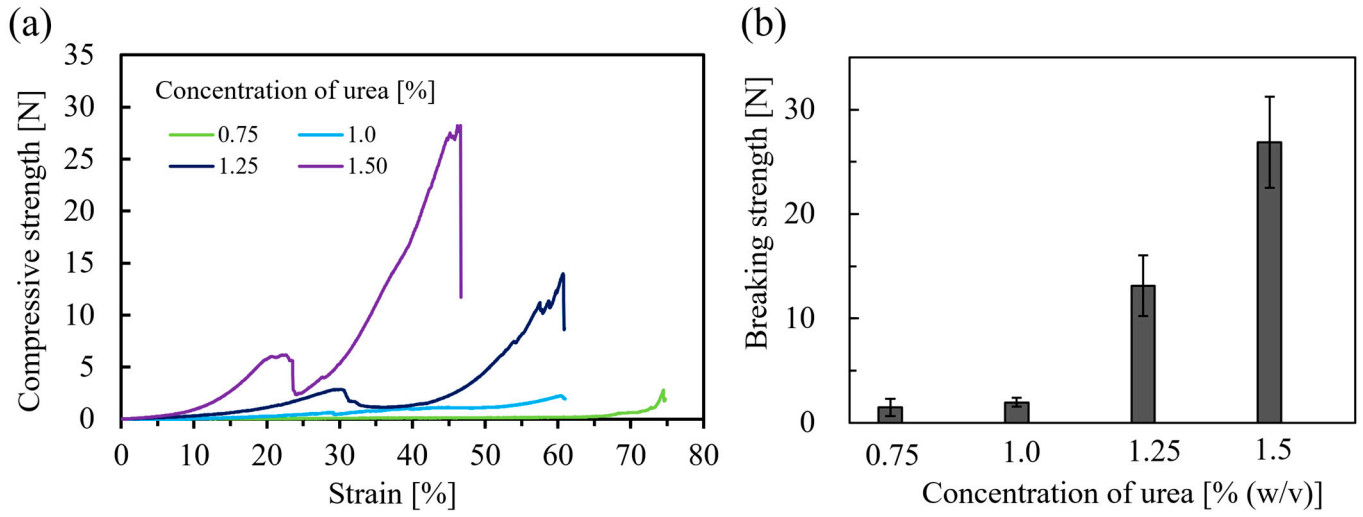


Figure 4. The mechanical properties of chitosan hydrogels prepared by autoclaving chitosan aqueous solutions with different concentrations of urea (0.75–1.5% (*w*/*v*): condition d–g). (**a**) typical strain-compressive strength curve of the chitosan hydrogels, and (**b**) their breaking strength (n = 4).

3.4. Investigation of Whether the Chitosan Hydrogels Require Washing

In the present study, we proposed a one-step method to achieve pH neutralization, hydrogelation, and sterilization of a chitosan aqueous solution using the hydrolysis reaction of urea by autoclaving. Here, it is important that the sterilized chitosan hydrogel does not require washing to remove unfavorable additives. This is because washing the hydrogel requires a re-sterilization process after that, that is, the number of processes increases. Therefore, in order to show low toxicity of the hydrogel, we investigated the pH, osmotic concentration, and the total concentration of NH_3 and NH_4^+ in a hydrogel, as shown in Table 2. Chitosan hydrogels prepared under conditions of d and e were suitable for physiological condition (pH: 7.4, osmotic concentration: 285 mOsm/L). An intravenous administration of ammonium chloride corrective injection 5 mEq/mL (Otsuka Pharmaceutical, Japan) at doses of 0.17 mmol/min is accepted. When 10 mL of the hydrogel is implanted in the body, the rate of diffusion of NH_3 and NH_4^+ into body tissues would be expected to be lower than its rate of administration. Therefore, the hydrogels would be acceptable.

Table 2. The pH, the osmotic concentration, and the concentration of NH_3 and NH_4^+ of the chitosan aqueous solution and chitosan hydrogels.

	Concentration of Urea [% (*w*/*v*)]	pH (Pre-Autoclaving)	pH (Post-Autoclaving)	Concentration of NH_3 and NH_4^+ [mmol/L]	Osmotic Concentration [mOsm/L]
a	0.0	5.5 ± 0.0	5.5 ± 0.0	0	111
b	0.25	5.5 ± 0.0	6.0 ± 0.0	16	160
c	0.5	5.5 ± 0.0	6.5 ± 0.0	51	219
d	0.75	5.5 ± 0.0	7.0 ± 0.0	83	276
e	1.0	5.5 ± 0.0	7.5 ± 0.1	103	329
f	1.25	5.5 ± 0.0	7.7 ± 0.0	106	372
g	1.5	5.5 ± 0.0	8.1 ± 0.0	115	418

All chitosan hydrogels were prepared by autoclaving 2.5% (*w*/*v*) chitosan aqueous solutions for 20 min.

3.5. Cytotoxicity of the Chitosan Hydrogels

We examined the cytotoxicity of chitosan hydrogels prepared from chitosan aqueous solution containing 1% (*w*/*v*) urea (condition d) according to the procedure of a previous report [25,26]. Mouse fibroblast-like cell line L929 was used as a model of fibroblast cells. The absorbance of each medium is shown in Figure 5. There was no statistical difference between the growth medium containing chitosan hydrogels and the growth medium with no additives (negative control). The results show that there is extremely low cytotoxicity of non-washed chitosan hydrogels fabricated using the autoclave technique.

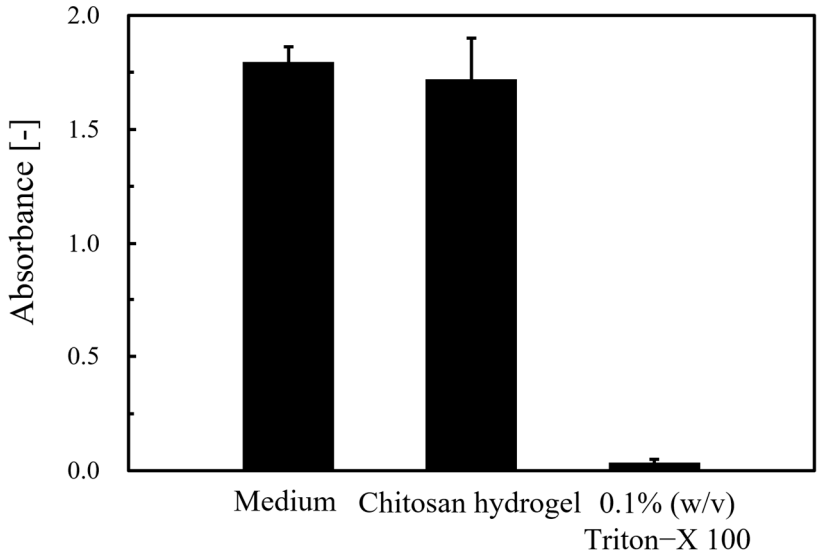


Figure 5. Cytotoxicity of chitosan hydrogels prepared under condition e (n = 5).

4. Conclusions

In this study, we prepared chitosan hydrogels in a one-step preparation method using hydrolysis of urea through autoclaving without any crosslinkers. The technique enables a chitosan aqueous solution to be pH-neutral, form a hydrogel, and be sterilized in one step. The swelling properties and mechanical properties of the hydrogels could be controlled by changing the urea concentration in the chitosan solutions. The chitosan hydrogels at concentrations of 0.75–1.0% (*w*/*v*) of urea were suitable for physiological conditions (pH: 7.0–7.5, osmotic concentration: 286–329 mOsm/L), and had acceptable concentration of ammonia (≤0.1 mmol/mL), which is a low-toxic biomaterial. Furthermore, not only pure chitosan, but also chitosan derivatives are used for hydrogels. Considering that other previous studies have shown that chitosan hydrogels that are pH-neutral and do not use any toxic crosslinkers are made from chitosan derivatives, the method is very practical in terms of manufacturing cost. Furthermore, the hydrogels would have excellent biological properties derived from chitosan, such as antibacterial, hemostatic, and wound healing properties, and thus would be used as hemostatic materials or wound dressings. It could

also be used as a tissue engineering scaffold for cell culture as well as other chitosan hydrogels. This research would provide a great foundation for the application of chitosan hydrogels to these biomaterials. In conclusion, the autoclave technique for preparing wash-free and non-toxic chitosan hydrogels has great potential.

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Appendix A

The hydrolysis rate of urea was calculated from the amount of OH^- produced in these reactions. The pH equation is $pH = -\log[H^+]$, that is $[H^+] = 10^{-pH}$, and then the amount of OH^- that contributed to the increase in pH in a chitosan aqueous solution is expressed by the following equation:

$$\text{The amount of } OH^- \text{ contributing to the increase in pH in a chitosan aqueous solution [mol]} = (10^{pHb} - 10^{pHa}) \times \frac{v}{1000} \quad \text{(A1)}$$

where pH_b and pH_a are the pH of chitosan aqueous solutions before autoclaving and the pH of chitosan hydrogels after autoclaving, respectively. v [mL] is the volume of chitosan aqueous solutions or chitosan hydrogels before or after autoclaving. Chitosan deprotonates its protonated amino groups when pH increases. Because the pKa of chitosan is 6.5, and $[Chi{-}NH_3^+]/[Chi{-}NH_2] = 10^{-pH+pKa}$, the rate of protonation of the amino groups of chitosan is described by the following equation:

$$\text{The rate of protonation of amino groups of chitosan [mol]} = \frac{10^{-pH+pKa}}{10^{-pH+pKa}+1} \times 100 \quad \text{(A2)}$$

In addition, according to (3), the amount of deprotonated amino groups of chitosan in a solution or a hydrogel is equal to the amount of OH^- that is calculated by the following equation:

$$\begin{gathered}\text{The amount of } OH^- \text{that contributed to the deprotonation of amino groups of chitosan [mol]} \\ = \frac{\text{The protonation rate at pHb} - \text{the protonation rate at pHa}}{100} \times \text{total amount of amino groups of chitosan}\end{gathered} \quad \text{(A3)}$$

Therefore, the amount of OH^- produced by the urea hydrolysis reaction is the sum of the amount of OH^- that contributed to the increase in pH of a chitosan aqueous solution and the amount of OH^- that contributed to the deprotonation of amino groups of chitosan as the following equation:

$$\begin{gathered}\text{The amount of } OH^- \text{ produced by urea hydrolysis reaction [mol]} \\ = \text{the amount of } OH^- \text{that contributed to the increase of pH of a chitosan aqueous solution} \\ + \text{the amount of } OH^- \text{that contributed to the deprotonation of amino groups of chitosan}\end{gathered} \quad \text{(A4)}$$

where the amounts of OH^- and NH_4^+ produced by the urea hydrolysis reaction are equal (Equation (2)). Because the pKa of NH_4^+ is 9.25, and $[NH_4^+]/[NH_3] = 10^{-pH+pKa}$, the total amount of NH_3 and NH_4^+ produced by urea hydrolysis reaction is calculated by the following equation:

$$\text{The total amount of } NH_3/NH_4^+ \text{ produced by urea hydrolysis reaction [mol]} = \text{The amount of } NH_4^+ \times \frac{10^{-pHa+pKa}+1}{10^{-pHa+pKa}} \quad \text{(A5)}$$

Thus, the amount of urea hydrolyzed is calculated by the following equation:

$$\begin{aligned}&\text{The amount of hydrolyzed urea calculated from the pH change [mol]}\\&= \text{The total amount of } NH_3 \text{ and } NH_4^+ \text{ produced by urea hydrolysis reaction} \times \tfrac{1}{2}\end{aligned} \quad \text{(A6)}$$

The rate of hydrolysis of urea after autoclaving of the chitosan aqueous solution was determined by the following Equation (4).

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