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Preparation and Characterization of Biodegradable Sponge-like Cryogel Particles of Chitosan via the Inverse Leidenfrost (iLF) Effect

Endang Ciptawati,* Hayato Takase, Nozomi Morishita Watanabe, Yukihiro Okamoto, Hadi Nur, and Hiroshi Umakoshi*

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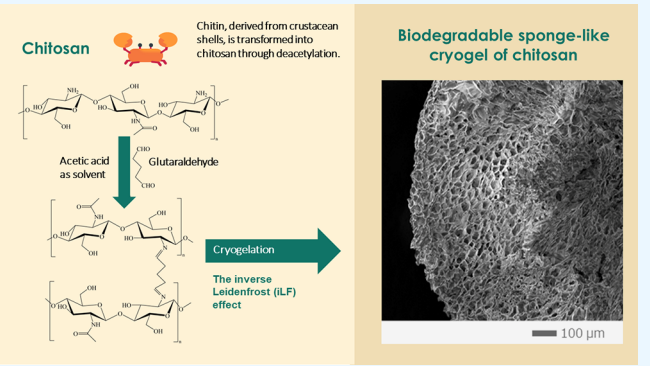
ABSTRACT: Chitosan-based cryogel particles were synthesized using the inverse Leidenfrost (iLF) effect, with glutaraldehyde employed as the cross-linker. The resulting cryogels exhibited a sponge-like morphology with micrometer-sized interconnected pores and demonstrated resilience, withstanding up to three compression-release cycles. These characteristics highlight the potential of chitosan cryogels for diverse applications, including adsorption and biomedical uses. We further investigated the influence of varying acetic acid concentrations on the properties of the chitosan cryogels. Our findings revealed that the particle size distribution of the cryogels ranged from 1300 to 2900 μm. As the concentration of acetic acid increased, the swelling degree of the chitosan cryogels decreased, stabilizing at an approximate value of around 6 at 0.03 mol of acetic acid. Additionally, the shift in the absorption peak of the OH and free amino groups from 3261 to 3404 cm^{-1} confirmed the cross-linking reaction between chitosan and glutaraldehyde.



INTRODUCTION

Natural polymers, also known as biopolymers, have received a lot of attention in the past decade as a "green" alternative to petroleum-based polymeric materials.[1] The massive amount of waste produced by nonbiodegradable synthetic plastics that pollute the environment is one of the primary reasons for searching environmentally friendly alternatives. Biopolymers cannot entirely replace synthetic polymers, but their use should be maximized as much as possible to take advantage of the benefits of their low toxicity and biodegradability.[2] The selection of biodegradable polymers is key to the advancement of modern biomaterials.[3]

Chitin is one of the most abundant natural polymers in the world, obtained from crustacean shells, and is used to produce chitosan through deacetylation. Chitosan, a natural cationic glycosaminoglycan, has distinctive properties, including biodegradability, bioactivity, and cross-linking ability. Additionally, this important biomaterial can be formulated into several platforms, such as, but not limited to, sponges and cryogels.[4−6] The usage of cross-linking agents for chitosan (glutaraldehyde, genipin, or tripolyphosphate) will increase its mechanical properties.[7−9] In acidic media, glutaraldehyde is highly reactive and is commonly employed to cross-link chitosan during cryogel formation.[10−12] Cross-linking reactions at low temperatures under cooling conditions have been proposed as a technique to prepare cryogels.

Cryogels have drawn intense attention in recent years due to their extraordinary properties.[13,14] For example, it is reported that they do not exhibit unwanted properties, such as brittleness, which is usually observed for macroporous gels made by phase separation polymerization. Cryogels are very tough and can resist extreme levels of deformation, such as elongation and torsion; they can also be squeezed almost completely without any crack propagation.[15] Cryogels produced by the cryotropic gelation method are macroporous hydrogels with a well-developed system of interconnected pores and shape memory. Cryogels are created at temperatures below the freezing point of the gelation solvent, where solvent crystals are produced in the semifrozen system. Subsequently, the reaction system is thawed, leaving micrometer-sized large pores.[16,17] Cryogels are also one of the most promising hydrogel-based biomaterials, a field that has been advancing rapidly. One of the ultimate characteristics of cryogels is their ease of synthesis, which can be accomplished using only aqueous solvent(s), making them suitable for various biological and biomedical applications.[18]

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The inverse Leidenfrost (iLF) effect is a phenomenon where the droplets (i.e., aqueous solution) are coated by a boiling film and become spherical when they move toward the surface of a bath of a cryogenic liquid with low boiling point (i.e, liquid nitrogen).[19] The procedure for synthesizing the cryogel particles can be summarized in the two following steps: preparation of frozen droplets by the iLF effect followed by cryogelation via frozen polymerization. To begin with, a polymer precursor is added dropwise into bulk liquid nitrogen (−196 °C), and then frozen droplets are made by the iLF effect, which are polymerized in liquid paraffine (−15 °C). After thawing and drying, the cryogel particles were obtained.[20]

Chitosan cryogel is a fascinating material that can help to reduce the use of petroleum-based materials. According to a previous study, chitosan cryogels are usually shaped as a monolith, sheet, particle, or disk. These materials are commonly used for biomedical applications such as tissue engineering and drug delivery systems.[21−24] In this work, we employed the iLF effect to produce chitosan cryogels. By using the iLF effect, smaller cryogel particles with sponge-like properties were successfully prepared under freezing conditions. These cryogel particles are more suitable for applications involving adsorption such as the bioremediation of heavy metals.

Herein, we report the preparation of biodegradable sponge-like cryogel particles of chitosan via the iLF effect and cryo-polymerization technique. This is the first reported case of chitosan cryogels prepared via the iLF effect. In this research, chitosan and glutaraldehyde were used as the backbone polymer and cross-linker, respectively. Since chitosan is insoluble in water, the most popular method is to use an acetic acid solution. In order to learn more about the structure−property relationships between the polymer and solvent as well as to optimize cryogel production, it can be interesting to first investigate how the acetic acid concentration affects the characteristics of the produced cryogels. In this study, the size distribution of cryogel particles was calculated from photographic images using ImageJ software. The swelling degree (SD) of the cryogels was investigated by gravimetry. Additionally, the Fourier transform infrared (FTIR) instrument was used to determine the changes in the functional groups of the material. The obtained cryogel particles with sponge-like structures were observed by scanning electron microscopy (SEM). Furthermore, the deformation and recovery behavior of both cryogel particles were observed using optical microscope images.

MATERIALS AND METHODS

Materials. Chitosan was purchased from Sigma-Aldrich (Japan). Acetic acid, ethanol, glutaraldehyde solution (25%), and liquid paraffin were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Ultrapure water (specific resistance 18.2 MΩ cm) was prepared with Direct-Q UV 3 (Merck, Osaka, Japan). All materials were used as received without further treatment.

Preparation of Cryogel Particles. The cryogel particles were created by polymerization under freezing conditions.[20,25,26] Ice crystals were used as porogens in the polymerization process after being templated in a polymer precursor. The cryogel particles in this study were created by using the droplet method and the iLF effect. Initially, 0.2 g of low molecular weight Chitosan was dissolved in 0.6 mL of 99.7% acetic acid solution (0.01 mol), followed by the addition of 10 mL of ultrapure water, and then the mixture was stirred by using a vortex. The cross-linker, 0.6 mL of a 25% (v/v) glutaraldehyde solution, was added, and the mixture was stirred. The cross-linking reaction between chitosan and glutaraldehyde is shown in Figure 1. Following that, the solution was transferred to a

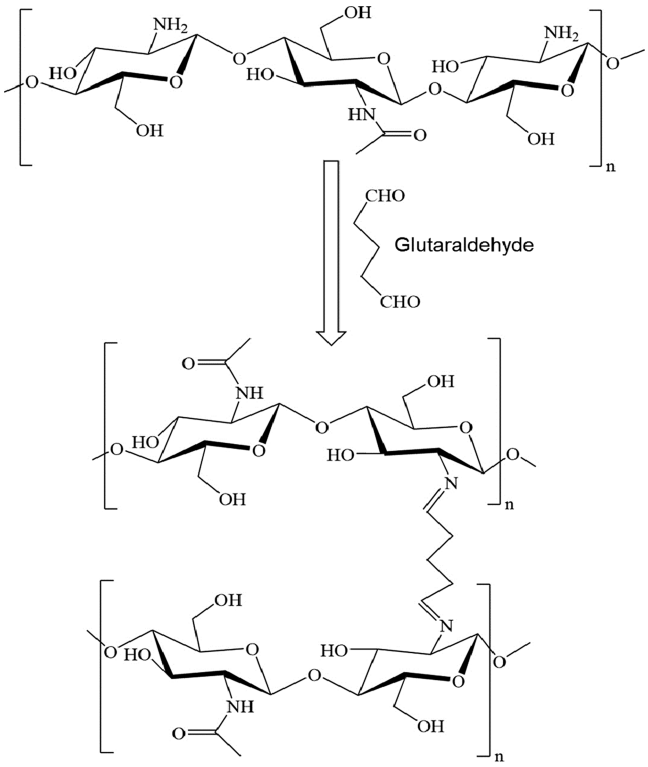

Figure 1. Illustration of chemical structure of cross-linking reaction between chitosan and glutaraldehyde.

syringe. The reactant solution was then dropped into liquid nitrogen from the syringe using a 25 gauge needle at a distance of about 20 cm above the base of the container. The fully frozen droplets were then transferred to liquid paraffin at −15 °C and cross-linked for 24 h. Afterward, the polymerized particles were thawed at 25−28 °C, and the residual cross-linker and paraffin were removed with ethanol, 1% NaOH solution, and ultrapure water. Finally, the cryogel particles were lyophilized for 24 h in order to obtain the dry cryogels. The schematic illustration of the preparation of chitosan cryogel particles can be seen in Figure 2.

Observation of Macroscopic Shape. The overall cryogel particles were observed as photographic images by a Samsung Galaxy A50 camera. The mean diameter of the particles (D_v) and standard deviation (σ) were calculated from the photographic images by using image J software. Furthermore, the coefficient of variation (C_v) was calculated according to eq 1:

$$C_v = \frac{\sigma}{D_v} \times 100\% \quad (1)$$

Characterization of Swelling Behavior of Cryogel Particles. The SDs of the cryogel particles determined their swelling behavior. The weights of the dried cryogel particles were measured to calculate the SDs. Initially, the dried cryogel particles were immersed in ultrapure water until they reached equilibrium at 25−28 °C; then, they were transferred into different containers, the ultrapure water on their surface was wiped off with filter paper, and their weights were measured. The SDs of the particles were calculated using eq 2:

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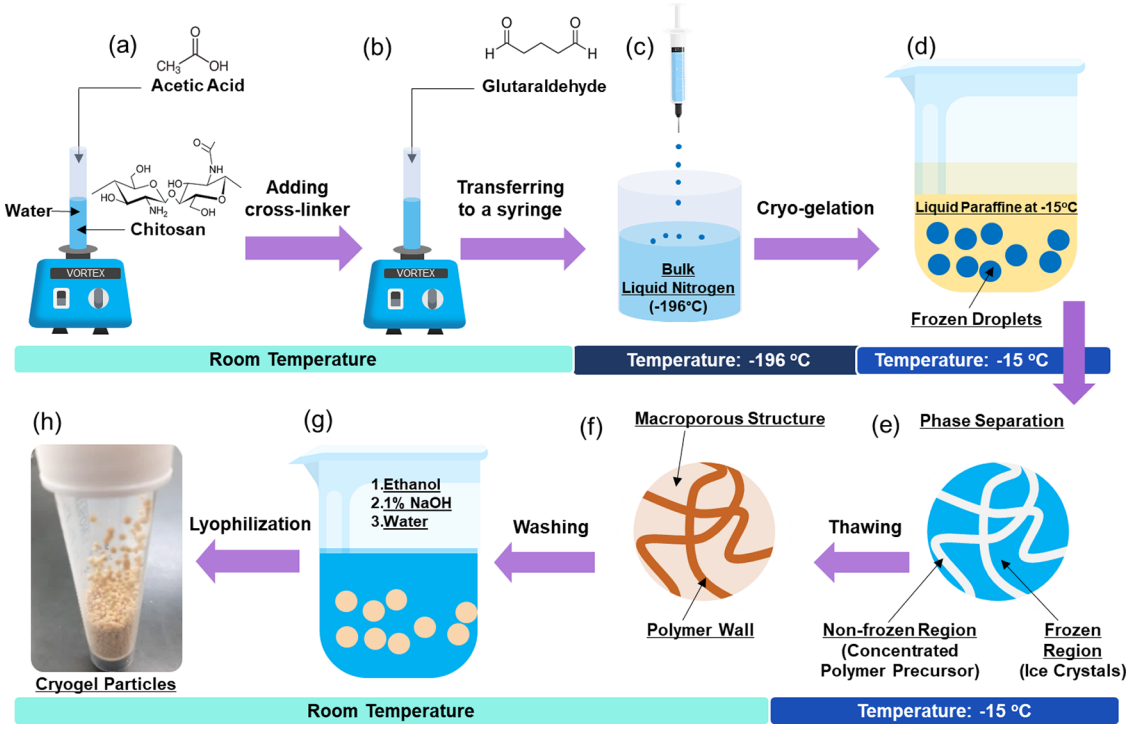

Figure 2. Preparation of chitosan cryogel particles via iLF cryo-method. (a) Dissolved chitosan in water/acetic acid solvent. (b) Adding cross-linker. (c) Dropped into liquid nitrogen to form a spherical particle shape. (d) Cryogelation. (e) Phase separation. (f) Monolithic super macroporous structure. (g) Purification. (h) Lyophilization.

$$SD = (W_{eq} - W_d)/W_d \quad (2)$$

where W_{eq} and W_d are the weights of the equilibrium and dried cryogel particles, respectively.

FTIR Analysis. The chemical functional groups of the materials, as well as their interactions with polymers and cross-linking agents, were examined using a FTIR spectrometer in the wavelength range of 400−4000 cm^{-1}.

Characterization of Cryogel Particle Morphologies. The cryogel particles, in general, had a monolithic structure with super macropores at the inner section. A scanning electron microscope was used to examine the morphologies of the cryogel particles (SEM, JEOL JCM-6000PLUS). The cryogel particles were cut with a cutter as a pretreatment step before being examined via SEM.

Characterization of Mechanical Properties of Cryogel Particles. By performing compression and swelling testing cycles, we are able to investigate the mechanical properties of cryogel particles. A cryogel particle was first placed in a micro syringe (Hamilton, 500 μL); it was then mechanically compressed, and its changing shape was observed using an optical microscope with a 4-objective lens. Its behavior and movement were video recorded correspondingly.

RESULTS AND DISCUSSION

Cryogel Particles of Chitosan. The chitosan cryogels were synthesized by the cryogelation of chitosan chains in the presence of glutaraldehyde as a cross-linking agent. Cross-linking agents are molecules with at least two reactive functional groups that create bridges between polymeric chains.[27] Furthermore, acetic acid was used as a solvent to dissolve chitosan. The nature of the cross-linking agent, polymer, solvent, and the reaction conditions (i.e., temperature, concentration, and duration) affect the structure and the chemical characteristics of the resulting chemically cross-linked network.[28] As a solvent, acetic acid might significantly impact the interaction between chitosan and glutaraldehyde. Moreover, due to the viscosity of the solution, the solvent could affect the characteristics and physical form of the cryogels. In this study, the acetic acid concentrations were adjusted and their effect on cryogel properties was analyzed.

The color of cryogel particles changed from white to yellow over time, indicating the formation of Schiff's base groups (cross-linking reaction).[11] The addition of glutaraldehyde caused the primary amino groups of chitosan to react with aldehyde groups in a condensation reaction, resulting in the formation of Schiff's bases.[27,29] Moreover, the change in viscosity of the solution after the addition of glutaraldehyde indicated the cross-linking reaction had happened.[11] The cryogel particles were prepared by combining the iLF effect and cryo-polymerization technique (iLF cryo-method).[20,25,26] Figure 3 shows the observation of cryogel particles with varied amounts of acetic acid (0.01, 0.02, 0.03, and 0.04 mol) as a frozen droplet, after cryogelation and lyophilization. As shown in Figure 3a3−d3, the cryogel particles had a spherical shape with a yellow color. After the diameter of individual particles from the images was observed, the size distribution was then determined. The size distribution of the particles was estimated using ImageJ software (Figure 3a4−d4). As a result, the cryogel particles, prepared by our iLF cryo-method, were distributed in the range of 1300−2900 μm.

The mean size of cryogel particles decreased as the concentration of acetic acid increased (Figure 4), due to the increased viscosity of the solution.[30] The distance between polymer chains was closer when the viscosity increased, resulting in denser and smaller cryogel particles. On the other hand, the coefficient of variation (C_v value) of cryogel particles increased as the amount of acetic acid in the solution increased. The C_v

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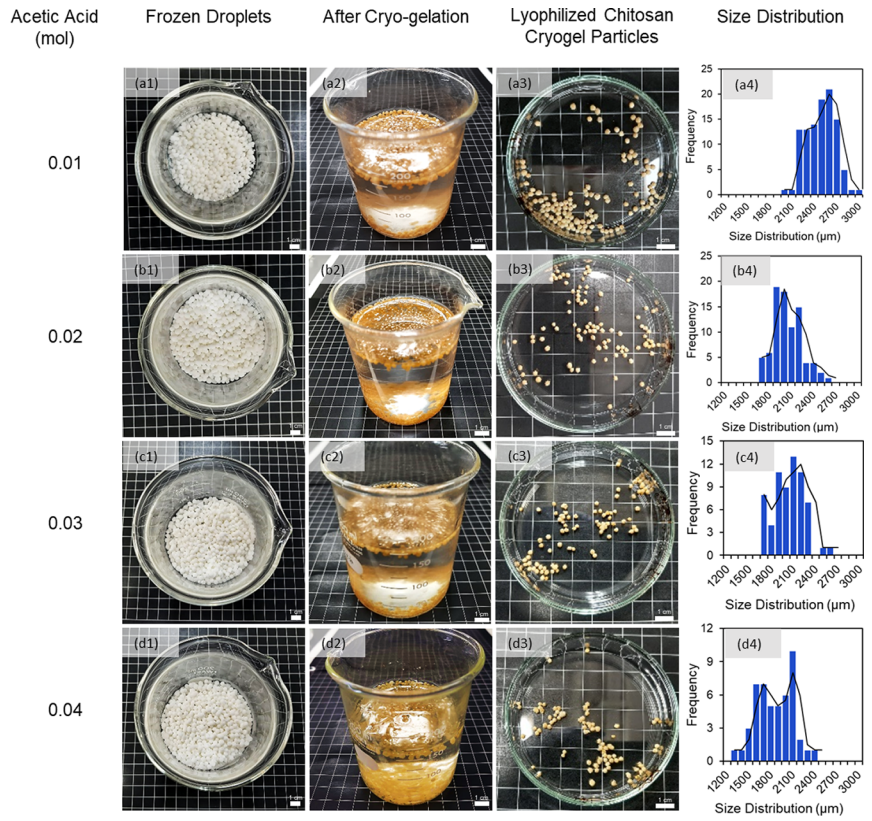

Figure 3. Observation of cryogel particles with varied amounts of acetic acid: (a) 0.01, (b) 0.02, (c) 0.03, and (d) 0.04 mol.

value is the standard deviation divided by the mean diameter. Even though there was an increase in the amount of acetic acid, the C_v values were less than 13%, indicating a narrow size distribution and high monodispersibility of the particle.

Swelling Behavior of Cryogel Particles. Water can easily enter the pores and inside the pore wall (polymer) regions of the cryogels because of their macroporous structure. Additionally, chitosan-based cryogels can successfully hold onto solvate moisture because chitosan is a highly hydrophilic polymer.[12] This allowed the cryogels to swell effectively. Cryogel pore wall thicknesses and cross-linking levels have been shown to affect the SDs of the cryogels; lower wall thicknesses and lower cross-linking levels result in higher SDs because the flexible pores make network expansion easier.[31]

According to Figure 5a, the SD of chitosan cryogels decreased as the amount of acetic acid increased. The increased acetic acid concentration results in the complete dissolution of chitosan. When the chitosan dissolved completely, the polymer's chains became closer to each other and arranged regularly, making it easier for the cross-linking reaction to occur. The greater cross-linking degree increased the cryogel's network density and caused a slow relaxation of the network chains. This circumstance has caused a more difficult network expansion, thus decreasing the SD.[32] When the amount of acetic acid was 0.01 mol, the SD of cryogels was the highest because the cross-linking reaction between chitosan and glutaraldehyde had not fully occurred due to the incomplete dissolution of the chitosan chain polymer. The SD remained relatively unchanged for 0.03 and 0.04 mol of acetic acid, marking that the SD had reached equilibrium and the cross-linking reaction had fully completed. Hence, we used 0.03 mol of acetic acid chitosan cryogels for the remainder of the study. In addition, as depicted in Figure 5b, the SD of cryogel particles 0.01 and 0.03 mol of acetic acid increased substantially during the first 15 min, then increased gradually until equilibrium was reached after about 1 h.

FTIR Analysis. The chemical structure of chitosan and the modifications made to the cryogel functional group by glutaraldehyde cross-linking were examined by using the FTIR spectra displayed in Figure 6. The characteristic peaks of

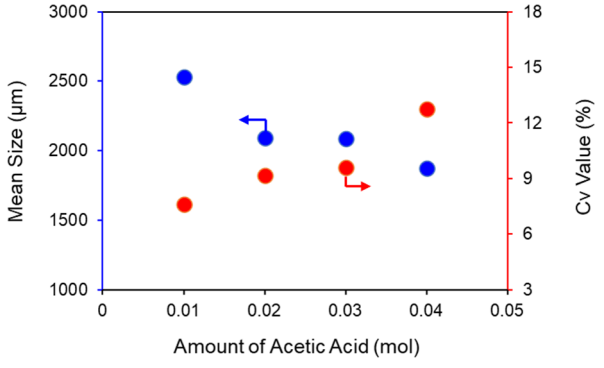

Figure 4. Mean size and C_V value of cryogel particles.

chitosan were seen at 2871 cm^{-1} (indicating the symmetric stretching of the C−H atoms), at 1550 cm^{-1} (showing amide N−H bending), and at 1033 cm^{-1} (attributed to (C−O−C vibration).[33,34] The cross-linking reaction of chitosan in the presence of glutaraldehyde was indicated by the Schiff base reaction, which was confirmed by infrared spectroscopy. The intensity and locations of the peaks were seen to change significantly. The free amino group (Amide A) and OH group's absorption peak shifted from 3261 to 3404 cm^{-1}.[34] The presence of the peak at 1648 cm^{-1} from the imine bonds N=C in

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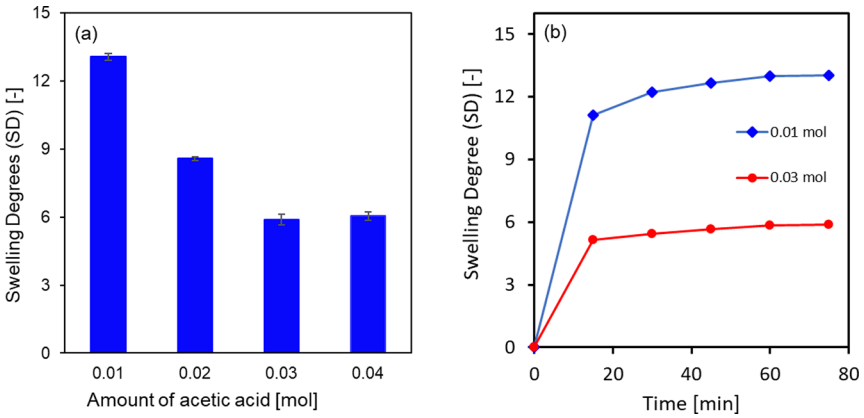

Figure 5. (a) Swelling degree of cryogel particles with different amounts of acetic acid. (b) Swelling degree of chitosan-glutaraldehyde cryogel particles with 0.01 and 0.03 mol of acetic acid over time.

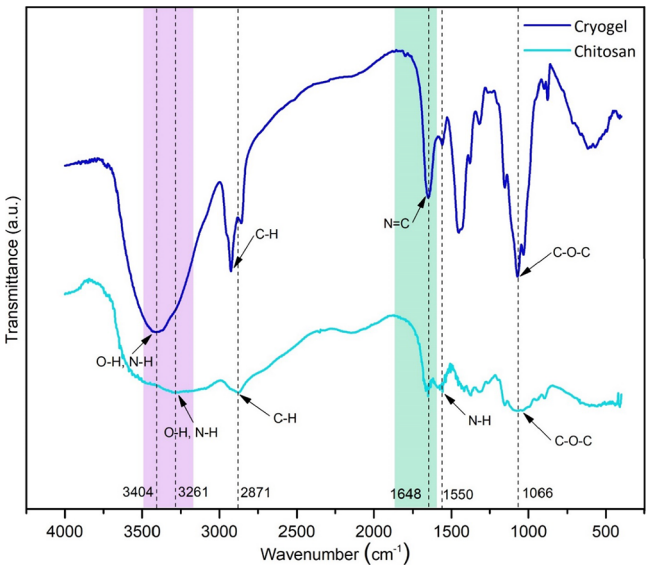

Figure 6. FTIR spectra of chitosan and 0.03 mol of acetic acid chitosan cryogel.

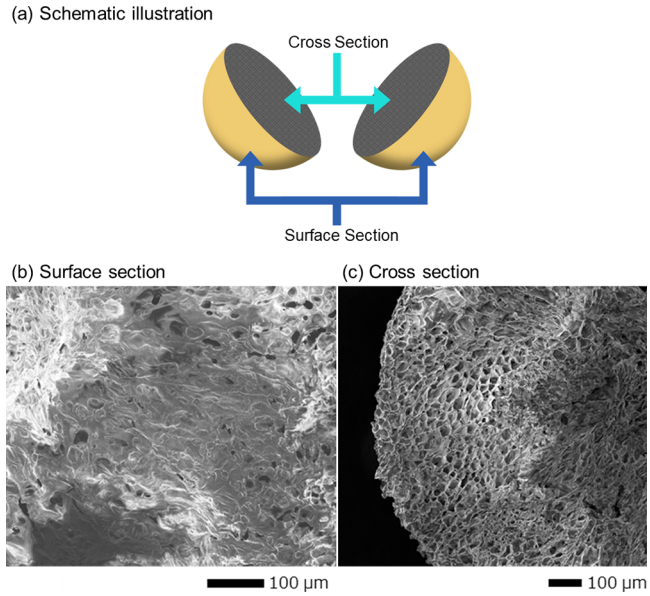

Figure 7. (a) Schematic illustration of surface section and cross section of cryogel particle; SEM images of 0.03 mol of acetic acid chitosan cryogel (b) surface section and (c) cross section.

cryogel spectra further demonstrated the cross-linking reaction between the aldehyde groups of glutaraldehyde and free amino groups of chitosan.[35] This reaction is favored under slightly acidic pH conditions,[12] which are provided by the presence of acetic acid, and are conducive to the formation of stable imine bonds (N=C).

Particle Morphologies. The morphology of 0.03 mol of acetic acid chitosan cryogel was determined by SEM analysis (schematically illustrated by Figure 7a) for the surface section (Figure 7b) and cross section (Figure 7c). The SEM results showed that the cryogels had sponge-like morphology and micrometer-sized interconnected pores. The use of acetic acid resulted in the dissolution of chitosan and created microporous cryogels with thin walls. The polymer concentration, processing settings, and drying process all affect the porousness of the cryogels.[36] The results showed that the porous structure of the cryogel particles on the surface is different from the cross section, with the surface being smoother and having fewer pores, while the cross section had more pores and a larger porous size. The pore diameter of chitosan cryogels at the surface was in the range of 3−24 μm, while the cross section pores had diameters ranging in size from 5 to 35 μm. The difference in the porous morphology between the surface and internal region was caused by the high viscosity.[37] During the preparation process, the iLF effect was used to coat the polymer precursor droplet with a boiling film of liquid nitrogen. This caused the droplet and nitrogen gas interfaces to experience shear stress. The shear stress causes the size of the porogen to become smaller. On the other hand, due to the high viscosity of the particles, shear stress cannot penetrate very deeply inside the cross section. Therefore, the porogen should be larger in the cross section. The porogen's size contributed to the pore's difference in size between the surface and cross section.[25]

Sponge-like Mechanical Properties. The deformation study of the cryogel particles produced here was examined by performing mechanical compression-release tests in a micro syringe, as shown in Figure 8a. The chitosan cryogel particles' shape drastically deformed when compressed mechanically, but it quickly regained its initial shape when the force was released (Figure 8b−d), exhibiting sponge-like reversibility.[20] The deformation behavior of the cryogel particles was documented on video during mechanical compression-release tests (Supporting Movies 1−4). Mechanical properties of cryogel particles are

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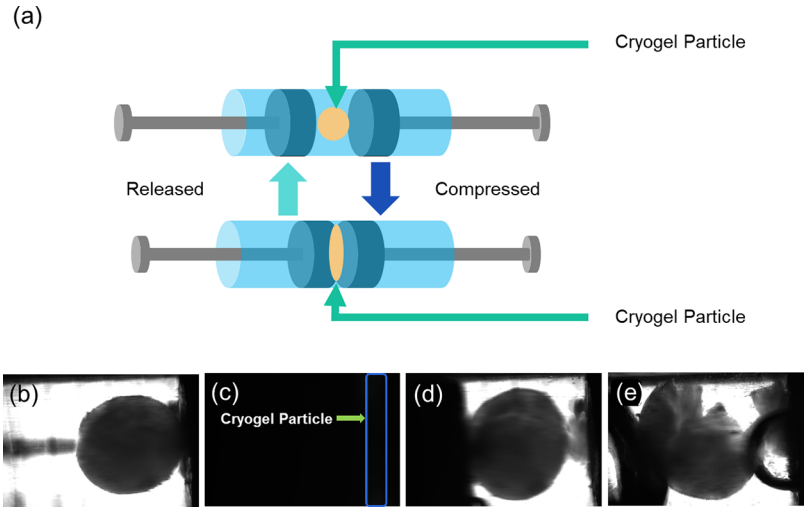

Figure 8. (a) Schematic illustration of compression and release of the cryogel particle. Optical microscope images of 0.03 mol of acetic acid chitosan cryogel: (b) before compression, (c) during compression, (d) after 1st compression, and (e) after 4th compression.

Table 1. Various Types of Natural Cryogels, Characteristics, and Applications

no.	monomer/ polymer	additive/cross-linker	cryogel type	size (mm)	swelling degree	reversibility	application	ref
1.	chitosan	glutaraldehyde	particles	2.1	5.8	positive	potential for adsorption and biomedical applications	this work
2.	chitosan	carboxymethyl cellulose	particles	n.d.	10	positive	drug delivery systems	21
3.	chitosan	glutaraldehyde, polypyrrole	particles	3	n.d.	n.d.	phthalate esters determination	39
4.	chitosan	diglycidyl ethers of ethylene glycol and polyethylene glycol	disks	25	3−17	positive	tissue engineering	6
5.	chitosan	$Na_2[PdCl_4]$, $K_2[PtBr_4]$, glutaraldehyde	monolith	7−9	low	n.d.	nitrophenol reduction	11
6.	chitosan	gelatin and glutaraldehyde	monolith	9	n.d.	positive	biocompatible scaffolds	22
7.	chitosan	difunctional polyurethane (DPU)	sheet	9.8	32	positive	scaffolds for tissue engineering	23
8.	chitosan	gluconic acid	disks	22	n.d.	n.d.	wound dressing	24
9.	chitosan	glutaraldehyde	monolith	n.d.	n.d.	n.d.	separation and enrichment of heavy metal ions	40
10.	chitosan	oxidized dextran and gelatin	monolith	7	High	positive	scaffolds for tissue engineering	41
11.	agarose	gelatin	particles	2.7	n.d.	positive	n.d.	25

related to the degree of cross-linking of cryogel particles. Cryogel particle resistance to the compression-release test increased with the degree of cross-linking (the amount of acetic acid). For example, at 0.01 mol of acetic acid, it could tolerate only one compression cycle; however, at 0.04 mol, it could withstand four cycles of compression. The 0.03 mol of acetic acid chitosan cryogel was crushed and lost its shape after the fourth compression (Figure 8e). This might have occurred because the chitosan cryogel particles were made from natural polymers with different mechanical properties compared to those of synthetic polymers. A similar result could also be found in agarose cryogels, where the shape of the particle changed after compression.[25] On the other hand, cryogel made from synthetic polymers like poly(2-hydroxyethyl methacrylate) could withstand up to 13 cycles without losing its original form.[20] One way to solve this issue is to chemically modify chitosan cryogels by making the chitosan polymer more negatively charged, which creates repulsion forces between the chains of the polymer. Optimizing the glutaraldehyde concentration is another option. Chitosan polymer chains contain functional groups with a lower positive charge when the glutaraldehyde concentration is too high, which reduces the repulsion force between polymers.

Table 1 presents a summary and comparison of various natural cryogel types with respect to the type of monomer or polymer, additive, cross-linker, properties of the obtained materials, and application. The first 10 cryogels in Table 1 are chitosan-based (larger than the cryogels in this work) and shaped like a particle, monolith, disk, or sheet. The last cryogel, however, is another natural polymer (gelatin) made with the same technique as this study (iLF cryo-method) and of comparable size. Results showed the potential of chitosan cryogel particles for a variety of potential applications (i.e., adsorption and biomedical applications) based on the properties (i.e., size, SD, and reversibility) of chitosan cryogels in this work compared with the properties of cryogels in the table. For example, in the bioremediation of heavy metals, smaller particle sizes give more benefit to the adsorption process. With the smaller size of the particles, more metals can be adsorbed due to the larger contact surface between the particle and the solution.[38]

■ CONCLUSIONS

We have successfully demonstrated the synthesis of porous chitosan cryogel particles using the iLF effect and cryo-polymerization technique. Cryogel particles, with a size distribution ranging from 1300 to 2900 μm, exhibited a sponge-like morphology with micrometer-sized interconnected pores, as revealed by SEM analysis. SD of the cryogels decreased

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with increasing acetic acid concentration, stabilizing at an approximate value of 6 at 0.03 mol of acetic acid. Cross-linking of chitosan in the presence of glutaraldehyde was suggested by the Schiff base reaction and was confirmed by FTIR spectroscopy. Cryogels demonstrated sponge-like properties, withstanding up to three cycles of compression-release testing. These findings highlight the potential of chitosan cryogel particles as versatile biomaterials for diverse applications, including adsorption and biomedical uses, warranting further exploration to realize their full commercial potential.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06639.

Mechanical compression-release tests of cryogel particle with 0.01 mol acetic acid (AVI)

Mechanical compression-release tests of cryogel particle with 0.02 mol acetic acid (AVI)

Mechanical compression-release tests of cryogel particle with 0.03 mol acetic acid (AVI)

Mechanical compression-release tests of cryogel particle with 0.04 mol acetic acid (AVI)

■ AUTHOR INFORMATION

Corresponding Authors

Endang Ciptawati − *Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531, Japan; Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Malang, Malang 65145, Indonesia*; Phone: +81-7-0904-35534; Email: endang.ciptawati.fmipa@um.ac.id; Fax: +81-6-6850-6286

Hiroshi Umakoshi − *Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531, Japan*; orcid.org/0000-0002-9241-853X; Phone: +81-6-6850-6287; Email: umakoshi.hiroshi.es@osaka-u.ac.jp; Fax: +81-6-6850-6286

Authors

Hayato Takase − *Department of Chemical Engineering, Graduate School of Science and Engineering, Kagoshima University, Kagoshima 890-0065, Japan*

Nozomi Morishita Watanabe − *Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531, Japan*

Yukihiro Okamoto − *Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560-8531, Japan*

Hadi Nur − *Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Malang, Malang 65145, Indonesia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06639

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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