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Observation of Calcium-Alginate Gel with Micrometer-sized Network Structure

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Abstract

The micrometer-sized network structure of a calcium-alginate hydrogel, which was synthesized from sodium alginate, calcium sulfate dihydrate, trisodium phosphate 12-hydrate, glycerol, and water, was observed using an optical microscope, cryogenic-scanning electron microscope, and an energy-dispersive X-ray spectrometer. For observation with the optical microscope, the calcium-alginate hydrogel was stained with Calcein, which emits green fluorescence by binding with calcium ions. Calcium ions act as a crosslinking agent in calcium-alginate hydrogels. For observation with the cryogenic-scanning electron microscope and energy-dispersive X-ray spectrometer, the hydrogel was frozen at -130 °C in a state containing water without drying or xerogelation. The optical microscope and cryogenic-scanning electron microscope observations revealed that the calcium-alginate hydrogel had a mixed structure of regions where large-scale gel networks and domains existed, and regions where they did not exist. The scale of each region was several hundred micrometers to several millimeters. In regions of the large-scale network structure, the domains were several tens of micrometers or less in size, and were surrounded by

networks that were several hundred nanometers or more thick. Fluorescence emission of Calcein as observed by the optical microscope and elemental analysis with the energy-dispersive X-ray spectrometer indicated that the large-scale network is composed of accumulated calcium-alginate networks. Energy-dispersive X-ray spectroscopy also indicated that calcium alginate exists in the large-scale domains and in regions where no large-scale network structure exists. We considered that calcium-alginate networks of molecular sizes with a well-known egg-box structure exist in the large-scale network structure and regions where no large-scale network structure exists, and that the water constituting the hydrogel is preserved in them.

Keywords: hydrogel, alginate, gel network, domain structure, cryogenic-scanning electron microscopy.

1. INTRODUCTION

Ultrasound phantoms to mimic human tissues are required to obtain the ultrasound properties of the human tissue, such as the ultrasound propagation velocity, attenuation coefficient, and acoustic impedance. The phantom is also required to have a softness similar to that of the human body. The phantoms are fixed by urethane rubber [1-7], polyvinyl alcohol [5,6], agar [3,4,6-8], gelatin [1-4,6], and condensed milk [8,9]. We have studied a laboratory-developed ultrasound phantom for the human soft tissue, which was constructed from sodium alginate, calcium sulfate dihydrate, trisodium phosphate 12-hydrate, glycerol, and water [10]. The ultrasound properties of the proposed phantom are closer to the properties of the human soft tissue, as shown in Table 1 [11-18], when the mixing ratio of sodium alginate, calcium sulfate dihydrate, trisodium phosphate 12-hydrate, glycerol, and water is in a 1.5:2:1:7.5:50 mass ratio. The proposed phantom can change its shape according to the mold in which it is placed, and its texture approximates that of the soft part of the human body, as shown in Figure 1.

TABLE 1 Ultrasound properties of human soft tissue and proposed ultrasound phantom.

	Propagation velocity	Attenuation coefficient	Acoustic impedance

	(m/s)	(dB/cm/MHz)	$\times 10^6$ (kg/m^2/s^2)
Human soft tissue	1530–1600	0.3–1.0	1.3–1.7
Proposed phantom *	1540 ± 35	0.51 ± 0.08	1.65 ± 0.09

* Mean and standard deviation of 40 phantoms.

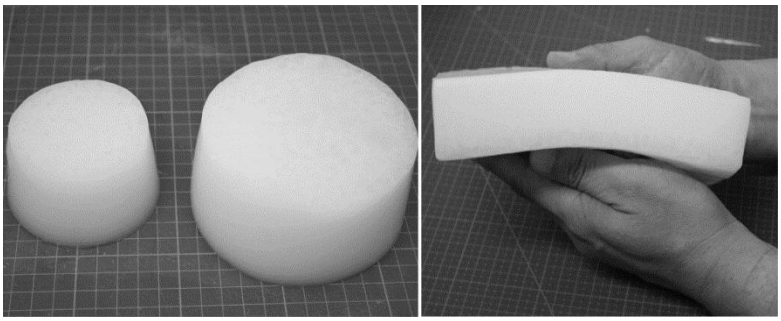

FIGURE 1 Left: Photograph of cylindrical phantoms with water capacities of 250 mL and 500 mL. Right: Rectangular parallelepiped phantom with water capacities of 1000 mL. The viscosity of the proposed phantom is approximately 30 Pa·s.

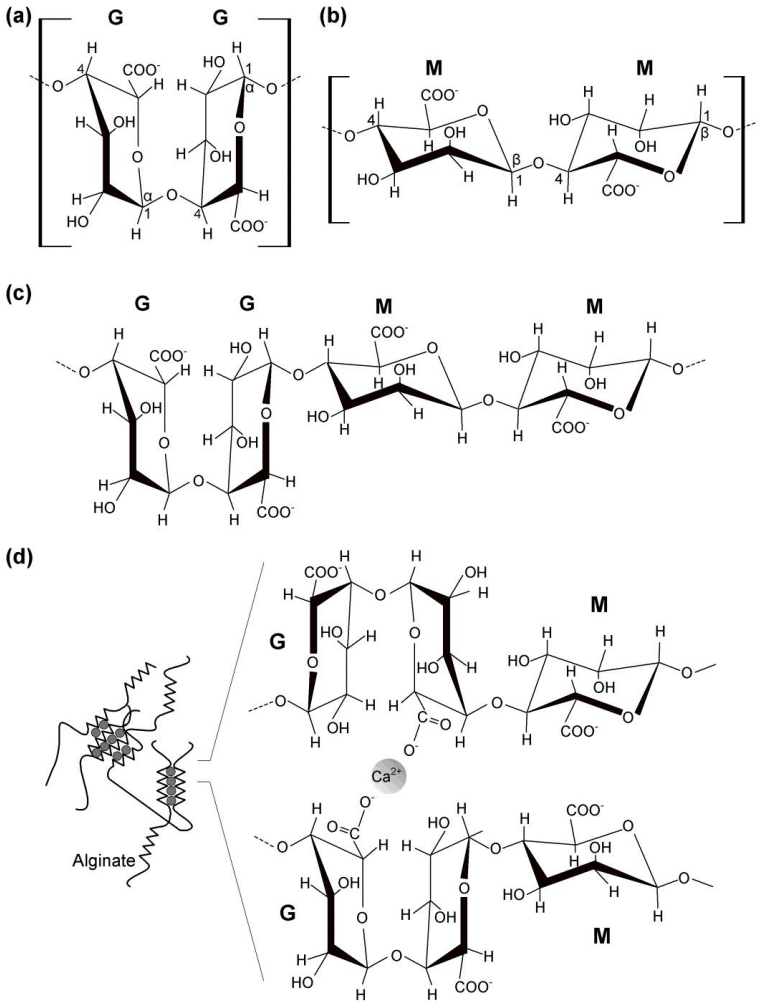

FIGURE 2 Chemical structure of alginates: (a) G–G blocks, (b) M–M blocks, (c) chain conformation with sequential arrangements of G–G–M–M blocks. (d) Illustration of cross-linked alginate network and calcium coordination of the egg-box model as described for the pair of G blocks in Ca^{2+} junction zones.

The proposed phantom is a calcium-alginate hydrogel with calcium added as a gelling agent to sodium alginate. Sodium alginate is a natural polysaccharide that is extracted from brown marine-algae seaweed. It is a linear unbranched copolymer of (1-4)-linked β-D-mannuronic acid (M block) and (1-4)-linked α-L-guluronic acid (G block) residues, which constitutes M, G, and MG sequential block structures, as shown in Figure 2(a)–(c). Calcium-alginate hydrogel is produced in an aqueous solution of sodium alginate that is cross-linked in the presence of multivalent cations, such as Ca^{2+}, and results in a flexible gel network with an egg-box structure, as shown in Figure 2(d). The calcium-alginate hydrogel network egg-box structure occurs when junction zones are formed due to the selective binding

of (1-4)-linked α-L-guluronic acid (G) residues to the calcium ions. The block-G regions are aligned side by side, resulting in the formation of a cavity, with calcium ions that link the chains to form a three-dimensional network [19-31]. Water is retained in the space that is formed by the molecular-sized calcium-alginate hydrogel networks.

In this study, the hydrogel network of the proposed ultrasound phantom was analyzed on the scale of micrometers by optical microscopy, cryogenic-scanning electron microscopy (cryo-SEM), and energy-dispersive X-ray spectroscopy (EDX). For optical microscopy observations, the proposed ultrasound phantom was stained with Calcein, which emits green fluorescence under exposure to an excitation light source in the presence of calcium ions (Ca^{2+}). Cryo-SEM is useful for studying the hydrogel structure containing water, as the conversion of the hydrogel to an aerogel is not mandatory [32-34]. Optical microscopy and cryo-SEM observations revealed that the proposed phantom gel prepared using calcium alginate contains a mixture of regions comprising large-scale domains that are surrounded by calcium-alginate networks and regions without large-scale domains and networks.

2. MATERIALS AND METHOD

2.1 Materials

The proposed calcium-alginate hydrogel was formed from sodium alginate with a viscosity of 500–600 cP, calcium sulfate dihydrate (98 %), trisodium phosphate 12-hydrate (98 %), glycerol (99.5 %), and water. These chemicals were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Sodium alginate is a gelling agent and calcium sulfate dihydrate is a curing agent. Trisodium phosphate 12-hydrate is a gelation retarder and a pH adjuster for gelation. The proposed phantom gelates at pH 11–12 in solution before the gelation. Glycerol (1906.7 m/s [35]) adjusts the ultrasound propagation speed in the phantom by mixing with water (1483 m/s [36]), and suppresses the generation of sodium-alginate lumps in the phantom.

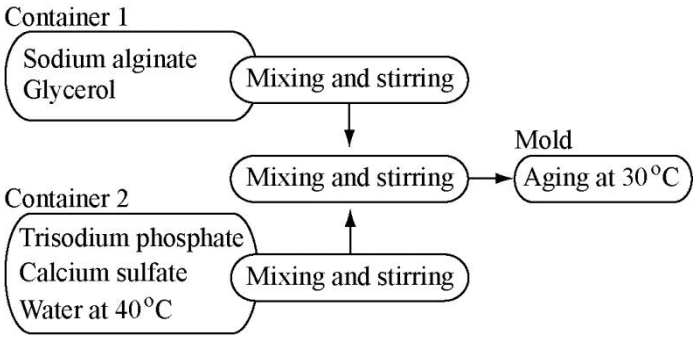

FIGURE 3 Fabrication of proposed phantom.

2.2 Phantom Fabrication

The proposed calcium-alginate hydrogel was prepared according to the procedure shown in Figure 3 [10]. Sodium alginate was mixed with glycerol, and stirred until the mixture became viscous. Calcium sulfate dihydrate and trisodium phosphate 12-hydrate were mixed with water at 40 ± 3° C. The latter mixture solution was poured into the former mixture, and was stirred until the mixture became viscous and uniform. Subsequently, the mixture was poured into a mold, and then placed in an incubator at 30 ± 3 °C for 24 h until the phantom gelled during aging. The proposed phantom mimics the human soft tissue the best when the mixing ratio of sodium alginate, calcium sulfate dihydrate, trisodium phosphate 12-hydrate, glycerol, and water is 1.5:2:1:7.5:50 by mass ratio.

2.3 Observation Methods

2.3.1 Optical Microscope Observation with Calcein

Calcium ions are important in the formation of calcium-alginate networks as a crosslinking agent, as shown in Figure 2. The distribution of calcium ions in the calcium-alginate networks was observed using a vertical-illumination-type optical microscope and Calcein. Calcein emits strong green fluorescence at 515 nm with an excitation light at 495 nm in the presence of calcium ions [37-40]. Calcium alginate was gelled in a petri dish as a mold, and the gelled calcium alginate was sliced thinly (to ~0.5 mm) as a sample for the optical microscopy observation. The sample was immersed at 30 °C for 24 h in a Calcein solution that was prepared by adding 0.05 g of Calcein to 100 mL of 50 % ethanol.

The Calcein used was purchased from Dojindo Molecular Technologies, Inc., Kumamoto, Japan. The microscope was attached with an optical filter that extracts excitation light from the light source of the microscope and an optical filter that extracts the fluorescence of Calcein.

2.3.2 Cryo-SEM Observation and Ultimate Analysis

Because a general sample observation method using SEM cannot observe hydrogels that contain a large amount of water directly under high vacuum, hydrogel dehydration is necessary; however, this may cause the sample to shrink and its structure to change. Cryo-SEM can be used to observe the hydrogel without dehydration, because the sample is fixed by rapid freezing, followed by fracturing before SEM observation. A sample from the phantom was observed using a cryo-SEM FEI Helios 600 NanoLab (Nanoscale Characterization and Fabrication Laboratory, Virginia, USA), and the ultimate analysis was conducted using an EDX Energy-XMAX 150 (Oxford Instruments PLC, Oxfordshire, UK). The freezing temperature was -130 °C, and the acceleration voltages for the SEM and EDX observations were 1.6 kV and 7 kV, respectively.

3. RESULTS AND DISCUSSION

3.1 Optical Microscope Observation

Figure 4 shows the optical microscopic images of the samples dyed with Calcein. Various shapes of the domains surrounded by the network are observed, as shown in Figure 4(a)–(d). Because the fluorescence from Calcein bound to calcium ions is observed from the network, we inferred that calcium alginate is aggregated in the network. The domain size is several tens of micrometers or less, and the network thickness is several hundred nanometers or more. The observed domains and networks were much larger than the networks comprising molecular chains of calcium alginate, such as those shown in Figure 2. A large domain surrounded by such a network will therefore simply be described as a large-scale network structure. Water is considered to be held inside the large-scale domains. Figures 4 (a)–(d) also indicate that the large-scale network structure disappears as the calcium-alginate network accumulates. Figure 4(e) shows the region where no large-scale network structure exists. Because water is held even when the large-scale network structure is not observed, water appears to be held by the molecular-sized networks shown in Figure 2, which cannot be observed with the optical microscope.

Figure 4(f) shows the boundary between the region where the large-scale network structure exists and the region where it does not exist. The two regions are smoothly connected and the large-scale network structure disappears.

The scale of each region was several hundred micrometers to several millimeters, as measured from the distance in the xy-direction of the microscope.

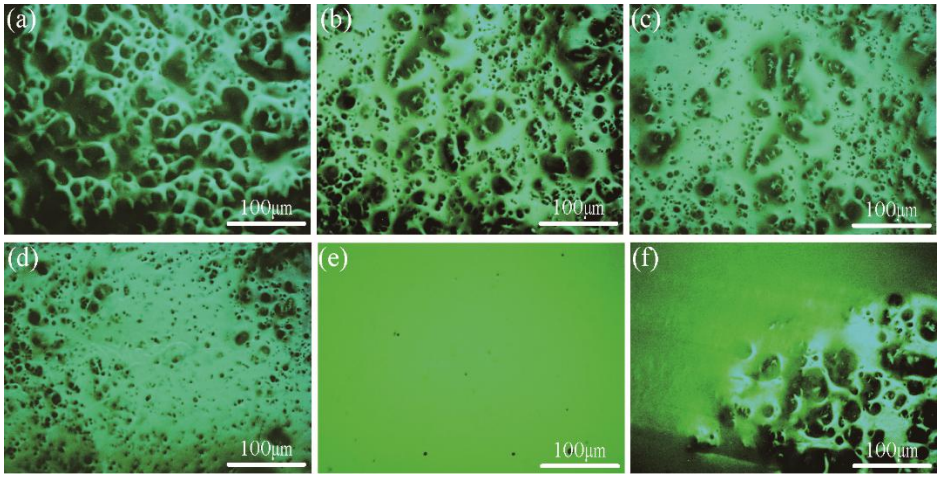

FIGURE 4 Optical microscopy images. Samples were stained with Calcein. (a)–(d) Various gel networks and domains. (e) Region in which gel networks and domains are not observed. (f) Boundary between the region where gel networks and domains are observed and the region where they are not observed.

3.2 Cryo-SEM Observation and EDX Analysis

Figures 5(a)–(c) show the various forms of the large-scale network structures that were observed using cryo-SEM, and 5(d)–(g) show the distribution of elemental Ca, Na, C, and P by EDX in the cryo-SEM image (c). The domain sizes are several tens of micrometers or less, and the network thicknesses are several micrometers or less. The elemental Ca and C, which are the primary elements of calcium alginate, are observed throughout the gel, but their concentrations are higher at the network. The networks are thought to be formed by the accumulation of calcium alginate. Na ions released from sodium alginate $(C_6H_7NaO_6)_n$ remain in the calcium-alginate network. P ions, which do not constitute the calcium-alginate hydrogel, are excluded from the networks.

Because Ca and C are also observed inside the domains where water is stored, the calcium-alginate networks are present inside the domain; however, the networks are considered to be of molecular size, as shown in Figure 2, and cannot be observed with the SEM resolution. In the optical microscopic observation using Calcein, we considered that the fluorescence from the inside of the large domains was not observed, owing to the performance limitations of our optical microscope.

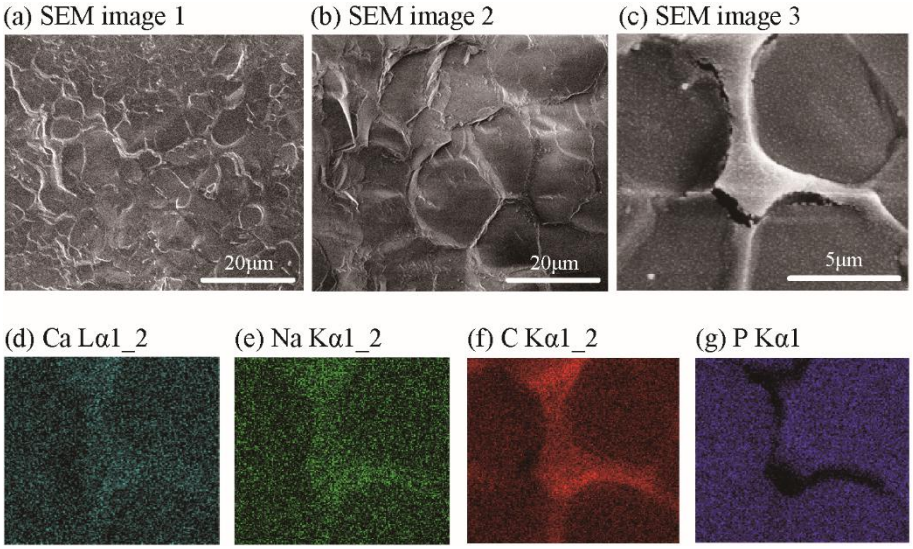

FIGURE 5 Cryo-SEM images and elemental distribution where the gel networks and domains are observed. (a)–(c) Cryo-SEM images, and (d)–(g) distribution of elemental Ca, Na, C, and P by EDX at networks and domains shown in cryo-SEM image (c).

Figure 6 shows the cryo-SEM image and the distribution of elemental Ca, Na, C, and P by EDX at the boundary between the left region where the large-scale network structure exists, and the right region where it does not exist. Figures 6(a)–(c) show the cryo-SEM images, and 5(d)–(g) show the distribution of elemental Ca, Na, C, and P by EDX in the cryo-SEM image (c). The domain sizes are several tens of micrometers or less, and the network thicknesses are several micrometers or less. Elements Ca, Na, C, and P are observed throughout the gel, but their concentrations are slightly higher at the network. It can be presumed that the calcium-alginate networks at the molecular size exist in the domain and in the region where the large-scale network structure is not observed, and that water is stored in them.

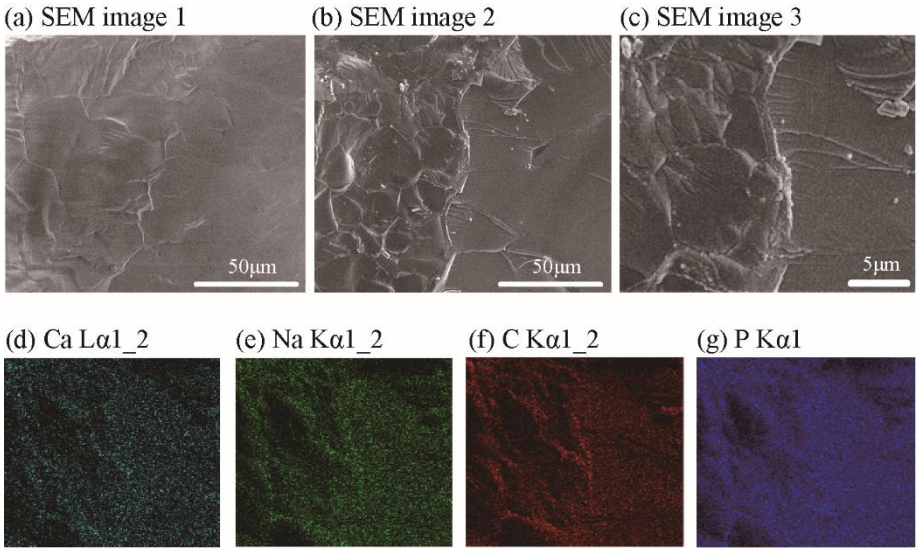

FIGURE 6 Cryo-SEM images and elemental distribution between the left region where the gel networks and domains are observed and the right region where they are not observed. (a)–(c) Cryo-SEM images. The cryo-SEM image (c) is an enlarged image of a part of the cryo-SEM image (b). (d)–(g) distribution of elemental Ca, Na, C, and P by EDX at networks and domains shown in cryo-SEM image (c).

3.3 Domain Shrinkage

Hydrogels generally lose water that has internally accumulated over time. Figure 7(a) shows the changes in the mass and appearance of the calcium-alginate hydrogels with a water capacity of 200 mL after they were placed in a constant-temperature incubator at 30 ± 3 °C. The proposed phantom rapidly lost moisture and shrunk, and only the unevaporated material remained. The mass of the phantom after 1250 h was approximately equal to that obtained by subtracting the mass of water that was initially present from the initial mass of the phantom.

Figure 7(b) shows the cryo-SEM images of the domains of the stored at room temperature for 144 h. The regions where the large-scale network structure does not exist are not observed, the domain sizes change small, and some parts that are considered to be domains in the initial state appear to shrink like beads. These changes indicate that water is stored inside the large-scale network structure and within the region where the large-scale network structure does not exist. Water appears to be held by the molecular-sized networks shown in Figure 2, which cannot be observed with the SEM resolution.

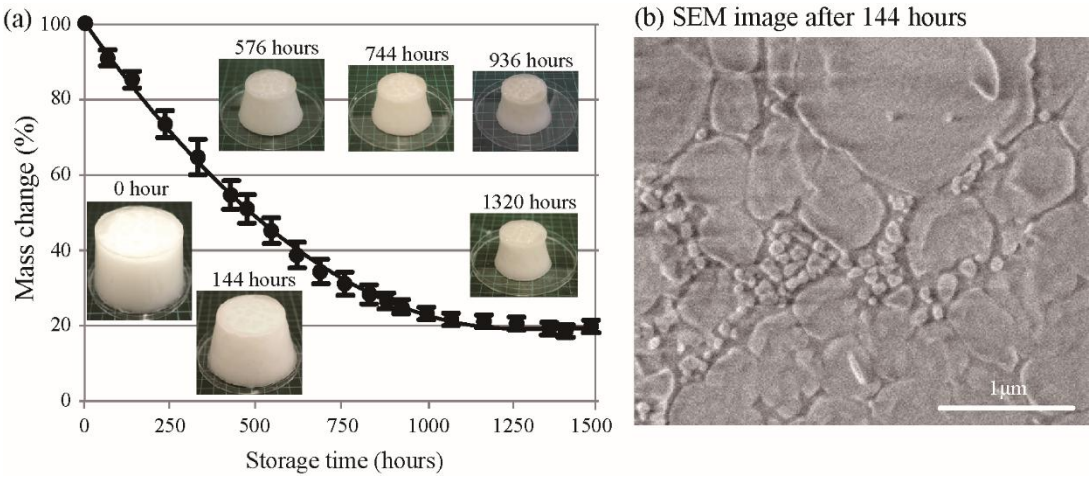

FIGURE 7 (a) Mass change of 200 mL of the phantom at 30 °C. Data show the mean value and standard deviation for the mass changes of 12 phantoms. The images in the graph show the appearance of the same phantom. Figure (b) is a Cryo-SEM image of the sample stored at room temperature for 144 h.

4. CONCLUSIONS

The structure of the calcium-alginate hydrogel made from sodium alginate, calcium sulfate dihydrate, trisodium phosphate 12-hydrate, glycerol, and water was analyzed by optical microscopy, cryo-SEM, and EDX. Observations with the optical microscope and cryo-scanning electron microscope show that the calcium-alginate hydrogel coexisted with the region where the large-scale network structure exists and the region where the large-scale network structure does not exist. The large-scale network structure is formed by the domains and the networks surrounding the domains. The domain size is several tens of micrometers or less, and the network thickness is several hundred nanometers or more. The large-scale network structure is much larger than the networks comprising molecular chains of calcium alginate. The elemental distribution as measured by EDX showed that Ca and C, which are the primary elements of calcium alginate, were distributed throughout the sample; in particular, their concentrations were higher at the network part. The networks are thought to be formed by the aggregation of calcium-alginate. A large amount of water is retained in the large domains and in the region where the large-scale network structure does not exist. We consider that calcium-alginate networks of molecular size are formed in the large-scale network structure and in the regions where the large-scale network structure cannot be observed.

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