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Autoclavable physically-crosslinked chitosan cryogel as a wound dressing

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Moist wounds were known to heal more rapidly than dry wounds. Hydrogel wound dressings were suitable for the moist wound healing because of their hyperhydrous structure. Chitosan was a strong candidate as a base material for hydrogel wound dressings because the polymer had excellent biological properties that promoted wound healing. We previously developed physically-crosslinked chitosan cryogels, which were prepared solely by freeze-thawing of a chitosan-gluconic acid conjugate (CG) aqueous solution, for wound treatment. The CG cryogels were disinfected by immersing in 70% ethanol before applying to wounds in our previous study. In the present study, we examined the influence of autoclave sterilization (121°C, 20 min) on the characteristics of CG cryogel because complete sterilization was one of the fundamental requirements for medical devices. We found that optimum value of gluconic acid content of CG, defined as the number of the incorporated gluconic acid units per 100 glucosamine units of chitosan, was 11 for autoclaving. An increased crosslinking level of CG cryogel on autoclaving enhanced resistance of the gels to enzymatic degradation. Furthermore, the autoclaved CG cryogels retained favorable biological properties of the pre-autoclaved CG cryogels in that they showed the same hemostatic activity and efficacy in repairing full-thickness skin wounds as the pre-autoclaved CG cryogels. These results showed the great potential of autoclavable CG cryogels as a practical wound dressing.

[**Key words:** Cryogel; Autoclave; Sterilization; Wound dressing; Chitosan]

Moist wounds are known to heal more rapidly than dry wounds (1). Hydrogel wound dressings are suitable for the moist wound healing because of their hyperhydrous structure. Various natural and synthetic polymers have been used as hydrogel dressing materials (2).

Chitosan is a naturally-derived aminopolysaccharide composed of repeating units of D-glucosamine and N-acetylglucosamine. This polymer has excellent biological properties, including biodegradability, biocompatibility and hemostatic and antimicrobial activities (3–5). Furthermore, chitosan promotes wound healing by stimulating inflammatory cells, including polymorphonuclear leukocytes (PMN) and macrophages (6). These properties of chitosan show that chitosan hydrogel has great potential as a wound dressing for moist wound healing. However, conventional chitosan hydrogels display an acidic state because the polymer is soluble only in acidic liquid due to its rigid crystal structure (4). Furthermore, these hydrogels often contain toxic additives (crosslinkers, polymerization initiators and high concentration of salts) and/or proteins of non-human origin that serve as antigens (7–12). These characteristics limit their application as a wound dressing. We previously reported that chitosan-gluconic acid conjugate (CG) is soluble in a neutral aqueous solution and the neutral CG solution forms a cryogel without additives (13). This is a first report concerning a new type of physically-crosslinked cryogel except for poly(vinyl alcohol) (PVA) cryogel. The neutral and physically-crosslinked CG cryogel displays biodegradability and high biocompatiblity, and has been used for cartilage and bone tissue engineering (14,15). Further, CG cryogel greatly accelerates wound healing because CG retains the favorable biological properties of chitosan for wound healing (13,16). In our previous studies, CG cryogel was disinfected using 70% ethanol solution before its application in wound care.

The purposes of the present study was to examine the influence of autoclave sterilization on the characteristics of CG cryogel. The Center for Disease Control defines sterilization as a process that destroys or eliminates all forms of microbial life, while disinfection (for example, treatment with 70% ethanol) describes a process which eliminates many or all pathogenic microorganisms, except bacterial spores, on inanimate objects (17). Before application to patients, complete sterilization (not merely disinfection) is one of the fundamental requirements for medical devices to prevent infection (18). Sterilization methods include autoclaving (steam sterilization), gamma-ray irradiation, ethylene oxide gas, ozone and peracetic acid exposure (17–20). Amongst these, autoclaving is simpler, safer and more environmentally friendly, because it does not require highly expensive devices or toxic chemicals and does not produce toxic byproducts (21). Therefore, autoclaving is currently considered the most practical means of sterilizing medical materials (22). However, it cannot be applied for materials that

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have low thermal resistance. Considering the thermal properties of the physically-crosslinked CG cryogels, we anticipated that CG cryogel has a high thermal resistance enough for withstanding autoclaving (see the results and discussion section).

MATERIALS AND METHODS

Materials PVA (degree of saponification 98%, degree of polymerization 2000) and egg-white lysozyme were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CG was synthesized by incorporating gluconic acid into chitosan through condensation of the carboxyl groups of gluconic acid and amino groups of chitosan using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide (13). Colloid titration was used to determine gluconic acid content, defined as the number of the incorporated gluconic acid units per 100 glucosamine units of chitosan (23). In the present study, we used three types of CG with different gluconic acid contents. CG with the content of 11, 20 and 42 were denominated as CG11, CG20 and CG42, respectively.

Preparation of cryogel and autoclaving CG was dissolved in a dilute HCl solution (pH 4.0) with dissolved 0.85% (w/v) NaCl. The pH was adjusted to 7.0 by gradually adding 0.1–1.0 M NaOH solutions to the CG solution, giving a final CG concentration of 2.0% (w/v). The CG solution (1 ml) was poured into a cylindrical mold (base diameter: 15 mm), frozen at −30°C for 12 h and thawed at room temperature to form CG cryogels. The cryogels were removed from the molds, immersed in excess saline in tightly-sealed glass vials and autoclaved (121°C, 20 min).

PVA was dissolved in hot distilled water at the concentration of 5.0% (w/v). The PVA solution was frozen and thawed under the same conditions as the CG solution. The PVA cryogels were also autoclaved in saline (121°C, 20 min).

Volume change of cryogels before and after autoclaving was determined by measuring the diameter and height of cryogels. Dry weight change of cryogels was also determined by measuring dry weight of hydrogel after careful rinse with distilled water and subsequent vacuum drying. X-ray diffraction (XRD) patterns of cryogels were recorded using an X-ray diffractometer (RINT2200 PCH/KG, Rigaku Co., Tokyo, Japan). Freeze-dried cryogels were ground into a powder, mixed with KBr and compressed into a disk. Fourier transform infrared (FT-IR) spectra of the samples were recorded using an IRT-3000 spectrometer (JASCO Corporation, Tokyo, Japan).

Enzymatic degradation of CG cryogels CG cryogels prepared by freeze-thawing of CG solution (0.5 ml) in a cylindrical mold (base diameter: 15 mm) were immersed in calcium and magnesium ions-free phosphate buffered saline (pH 7.4) containing 0.3 mg/ml lysozyme and gently shaken at 37°C. The wet weight of the cryogels was determined at predetermined intervals.

Blood clotting test We used a modified blood-clotting test to examine hemostatic properties of CG cryogel skeleton (24). Briefly, blood was obtained from the abdominal aorta of male Wistar rats (6 weeks old) under anesthesia using pentobarbital. Coagulation was prevented by mixing the whole blood with 109 mM sodium citrate aqueous solution (9:1 (v/v)) at 37°C. The whole blood (50 μl) was dispensed on pre-warmed CG and PVA sponges (37°C, 10 mm × 8 mm × 4 mm), which were prepared by instantaneous freezing in liquid nitrogen and drying under vacuum of the cryogels, followed by the addition of 5 μl 0.2 M calcium chloride aqueous solution. After incubation at 37°C for 5 min, the sponges were immersed in 6.25 ml distilled water and incubated at 37°C with shaking at 30 rpm for 10 min. Red cells that were not entrapped in clots were hemolyzed in distilled water. The concentration of hemoglobin released from hemolyzed red cells in water was determined by measuring the absorbance at 540 nm. The absorbance of distilled water directly added to the same volume of whole blood was used as the reference. The blood-clotting index (BCI) was determined by the following equation:

$$\text{BCI index} = \text{absorbance of sample} \times 100/\text{absorbance of reference} \quad (1)$$

All animal experiments were performed in accordance with the recommendations of Kagoshima University's "Guide for the Care and Use of Laboratory Animals".

Implantation of CG cryogels CG cryogels (2 ml) were sterilized by autoclaving as described above. As a control condition, CG cryogels were disinfected by immersing in excess 70% (v/v) ethanol with gentle shaking for 10 min and carefully rinsed with saline. These cryogels were subcutaneously implanted into three male DDY mice (7 weeks old) under anesthesia using pentobarbital. The mice were sacrificed after 1 week using an overdose of anesthetic. The area of the implant was dissected, fixed in 10% (w/v) neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Treatment of wounds with CG cryogels CG cryogels (2 ml) were prepared in a cylindrical mold (base diameter: 23 mm) by freeze-thawing of CG solution. The cryogels were treated by autoclaving or with 70% (v/v) ethanol. Dorsal skin of male Wistar rats (8 weeks old) was shaved and treated with 70% (v/v) ethanol for disinfection under anesthesia using pentobarbital. Two full-thickness skin wounds of 10 mm diameter were created in each rat and one of each wound was covered with a cryogel treated by autoclaving or with 70% (v/v) ethanol. The wounds were further covered with a second dressing (BFR (adhesive waterproof film), Nichiban, Tokyo, Japan). All dressings were fixed using an elastic adhesive bandage (Elastpore, Nichiban). The wound area was determined every alternate day. At 2, 4, 6, 8, 10 days postwounding, rats were sacrificed, and the skin, including the entire wound with adjacent normal skin, was excised for histological examination.

Statistical analysis Data are presented as the mean ± standard deviation. Statistical differences between two groups were analyzed using the two-tailed Student's unpaired *t*-test. Statistical differences among multiple groups were analyzed using the one-way analysis of variance (ANOVA) with Bonferroni analysis.

RESULTS AND DISCUSSION

The mechanism for cryogelation of CG is similar to that of PVA (13,25,26). The difference between PVA and CG cryogels is because they exhibit temperature-dependent solubility (PVA) and temperature-independent/pH-dependent solubility (CG) in water, respectively. The detailed cryogelation mechanism of CG is as follows (13). Unmodified solid chitosan hardly dissolve in neutral and alkaline water due to its rigid crystal structure, even at a high temperature (temperature-independent solubility). By contrast, unmodified chitosan readily dissolves in acidic water by electrical repulsion between protonated amino groups in the molecules (pH-dependent solubility). Because CG partially retains the high crystallinity of unmodified chitosan, it also has the temperature-independent/pH-dependent solubility in water (13). Difference between unmodified chitosan and CG is that CG dissolved in acidic water retains its solubility even after adjusting the pH of the solution to 7.0 though chitosan dissolved in acidic water forms precipitate at pH 7.0. On lowering the temperature of neutral CG solution, ice crystals are generated and grow in the solution. The growth of the ice crystal decreases the volume of the unfrozen liquid phase surrounding the ice crystals. Furthermore, ice crystals grow while eliminating CG molecules, leading to a significantly higher concentration of CG in the unfrozen liquid phase. The high CG concentration promotes the formation of rigid chitosan crystals (physical crosslinking site) in the unfrozen liquid phase. During thawing of the frozen solution, neutral liquid water is generated by the melting of ice crystals. Chitosan crystals display very limited solubility in the neutral liquid water as described above, resulting in formation of cryogel. We anticipated that CG cryogels can be sterilized by autoclaving without their melting or collapse due to the temperature-independent solubility of CG in water.

Tolerance of CG cryogels to autoclaving We first examined tolerance of CG42 cryogel to autoclaving. The cryogel completely dissolved in saline on autoclaving with a probability of 70% ($n = 10$). Modification of chitosan with gluconic acid would inhibit formation of rigid chitosan crystal, which has high thermal resistance, by steric hindrance. This suggests that decreased gluconic acid content causes successful formation of CG cryogel with higher thermal resistance through enhanced formation of chitosan crystal. Therefore, we examined tolerance of CG20 and CG11 cryogels to autoclaving. CG20 cryogel completely dissolved in saline with a probability of 50% ($n = 10$). It should be highlighted that tested all CG11 cryogels withstood autoclaving ($n > 10$). The autoclaved cryogels shrank about 40% while retaining their initial shape (Fig. 1A–C). They turned to be more yellowish in color. The color change might be attributed to the shrinkage of the cryogel. Fig. 1D shows ratio of dry weight of cryogels to that of CG used for cryogel preparation. Minimum weight loss was observed for CG11 after freeze-thawing of the aqueous solution. On the other hand, significant weight loss was observed for CG42 after freeze-thawing. Further, residual dry weight of CG11 was statistically higher than that of CG42 after autoclaving. These results show that CG cryogel with lower gluconic acid content displays higher degree of crosslinking than cryogel with higher content. The

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