Pressure-Induced Molecular Assembly of Hydrogen-Bonded Polymers

SHINGO MUTSUO,[1] KAZUYA YAMAMOTO,[2] TSUTOMU FURUZONO,[3] TSUYOSHI KIMURA,[4] TSUTOMU ONO,[1] AKIO KISHIDA[4]

[1]Department of Material and Energy Science, Graduate School of Environmental Science, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan

[2]Department of Nanostructured and Advanced Materials, Graduate School of Science and Engineering, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan

[3]Department of Biomedical Engineering, National Cardiovascular Center Research Institute, Fujishiro-Dai, Suita, Osaka 565-8565, Japan

[4]Department of Applied Functional Molecules, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Kanda-Surugadai, Chiyoda-Ku, Tokyo 101-0062, Japan

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ABSTRACT: Controlling the noncovalent bondings such as electrostatic interaction, van der Waals force and hydrogen bond, is the key factor to generate molecular assembly. We show that pressure is one of the most intensive variables for controlling these intermolecular forces and producing assembled structure. Macrogel and nanoparticles of hydrogen-bonded polymers were simply obtained through an ultrahigh-pressure process. The morphology of the obtained assembly depends on concentration and various conditions of the pressurization. These results indicate that the ultrahigh-pressure induces inter/intra-hydrogen bond, which is strong enough to maintain microassemblies such as gels and particles. This methodology leads to the molecular design of pressure-induced molecular assembly, and nonharmful processes for molecular separation and drug development. © 2008 Wiley Periodicals, Inc. J Polym Sci Part B: Polym Phys 46: 743–750, 2008
Keywords: crosslinking; hydrogels; nanoparticles; water-soluble polymers

INTRODUCTION

Molecular assembly technology has been gathering interest in the material processing field, especially nanotechnology. Molecular assembly is achieved by noncovalent bonding between adjacent molecules. The development of carbon nanotubes as circuit wires[1,2] and the incorporation of anticancer drugs and amphiphilic polymers into nanomicelles[3,4] are examples of molecular assembly in which noncovalent bonding, such as electrostatic interaction, van der Waals interactions and hydrogen bonds, are well combined.[5–8] Controlling these intermolecular forces is the key factor to create or collapse the assembled structure. Supramolecular chemistry has expanded to allow various elemental molecules to generate elegant assembles,[9–12] whereas the operative factors which regulate molecular assembly are mostly limited by the concentration and/or temperature. Here, we show that

Correspondence to: A. Kishida (E-mail: kishida.fm@tmd.ac.jp)

pressure, which is one of the most intensive variables in thermodynamics as well as the concentration and temperature,[13–16] can also be used for controlling the intermolecular forces to generate assembled molecules. We found that a poly (vinyl alcohol) (PVA) solution turned into a macrogel or nanoparticle through a simple ultrahigh-pressure process (10,000 atmosphere, 10 min). The morphology of the obtained assembly depended on the PVA concentration, indicating significant inter/intra-molecular hydrogen bonding. Our results demonstrated that ultrahigh-pressure induces hydrogen bonding in water, which is strong enough to maintain microassemblies such as gels and particles.[17,18] Since the interactive potential of molecules is brought out under ultrahigh-pressure, this technology would be applicable to realize the concept for designing assembly molecules proposed by Whitesides and coworkers.[19–21] Furthermore, this methodology leads to the molecular design of pressure-induced molecular assembly, and facilitates nonharmful processes for molecular separation and drug development.

EXPERIMENTAL

Materials

The degree of polymerization of the used PVA (Kuraray, Japan) was 1750. The degree of saponification was 99.8%.

Ultrahigh-Hydrostatic Pressurization

An aqueous PVA solution of predetermined concentration was poured into a plastic bag and was sealed. The bag solution was pressurized using an ultrahigh-pressure machine (hydrostatic pressure). The pressure was set to 1000–10,000 atmospheric pressures, and was processed over the predetermined time period.

Hydrogel Preparation by The Freeze-Thawing Method

An aqueous PVA solution was subjected to five cycles of freeze-thawing, in which the sample was frozen for 12 h at −20 °C, and then thawed for 12 h at 4 °C as one cycle. The mass change of the freeze-thawed sample and the high-pressure processed sample before and after soaking was measured, and the structures of the two gels, both of which had gel ratios over 90%, were compared.

Dynamic Light Scattering Measurement

A 0.5 w/v % PVA solution was high-pressure processed for 10 min at 10,000 atm, and the sample was diluted to an appropriate concentration with ultrapure water, and was subsequently filtered with a 5-μm pore mesh. The particle size was then measured with DLS-7000 (Otsuka Electronics, Japan) using an Ar laser (λ = 488 nm, 75 mW).

Swelling Ratio Measurement

The PVA hydrogel prepared by pressurization was immersed in pure water at room temperature for 10 days and then freeze-dried. The swelling ratio of the PVA hydrogel was calculated as follows:

$$\text{Swelling ratio} = \frac{W_h - W_d}{W_d} \times 100$$

where W_h is the weight of hydrated gel after the dialysis and W_d is the weight of dried gel.

Scanning Electron Microscopy

Observation of PVA assembly was carried out using a scanning electron microscope, S-4700 (Hitachi High Technologies). Specimen for SEM observation was prepared as follows: After a hydrogel was freeze-dried, it was coated with a thin layer of Pt–Pd by the vacuum evaporation technique.

Differential Scanning Calorimetry

DSC measurement was carried out to reveal the melting temperature of PVA assembly. It was carried out at heating rate of 5 °C/min under a constant flow of nitrogen gas.

^{1}H NMR Measurement

The nongelled portion of the pressurized PVA was obtained by the dialysis of the PVA hydrogel. The ^{1}H NMR spectra was obtained by the measurement of the PVA sample dissolved in dimethyl sulfoxide (DMSO-d_6).

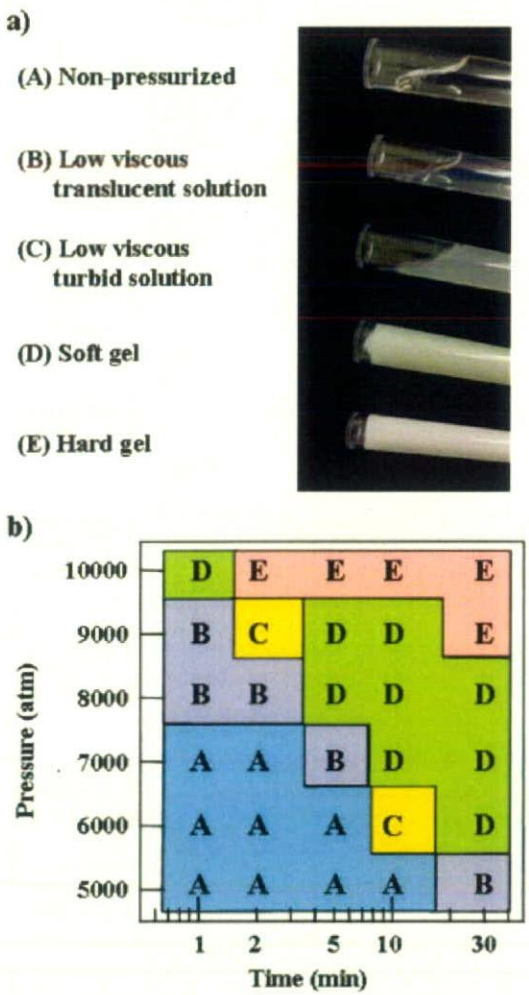

Figure 1. Pressure-induced PVA assembly. (a) Photographs of a 10 w/v% PVA solution pressurized under various conditions: (A) nonpressurized, (B) 7000 atm, 1 min, (C) 9000 atm, 1 min, (D) 7000 atm, 10 min, and (E) 10000 atm, 10 min. (b) Phase (constitutional) diagram of a 5 w/v % PVA solution pressurized under various conditions. The state was decided by visual observation according to the photographs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS AND DISCUSSION

PVA Assembly Formed by Pressurization

Aqueous solutions of PVA at 1–20 w/v % concentrations were pressurized hydrostatically under various conditions. Figure 1(a) shows photographs of typical samples of 10 w/v % PVA solutions pressurized at different atmosphere pressure (atm) for 10 min. A translucent solution, the precipitate and hydrogel of PVA was obtained by increasing the pressure, indicating that the assembly of PVA molecules was induced by pressure treatment. The hydrogel was stable in pure water, and the yield (gelation ratio) was 90% or more. It is well-known that PVA solutions transform into hydrogels when the solution was frozen and thawed sequentially several times; this procedure is called the freeze-thawing method. Approximately 10 days is required to form a hydrogel with similar strength as a hydrogel obtained by pressurization for only 10 min. Thus, this simple pressurizing method can be expected to be an energy saving process. The influence of the pressure conditions on the formation of a PVA assembly was examined using a PVA solution of 5 w/v % in detail. Figure 1(b) shows the state diagram of the PVA assembly in a pressure–time plot determined by visual observation according to the photographs shown in Figure 1(a). The translucent solution and hydrogel were acquired by pressure treatment at more than 8000 atm over a very short time (one min). The tendency for gelation of PVA with increasing pressure was observed for each step of pressurization. In addition, at constant pressure, a long period of pressure treatment induced assembly of the PVA, even in the case of only 6000 atm, and the hydrogel was obtained by pressurization for 30 min. Furthermore, DLS measurements of a 10 w/v % solution pressurized under conditions in which a hydrogel was not obtained revealed the formation of PVA nanoassembly and the

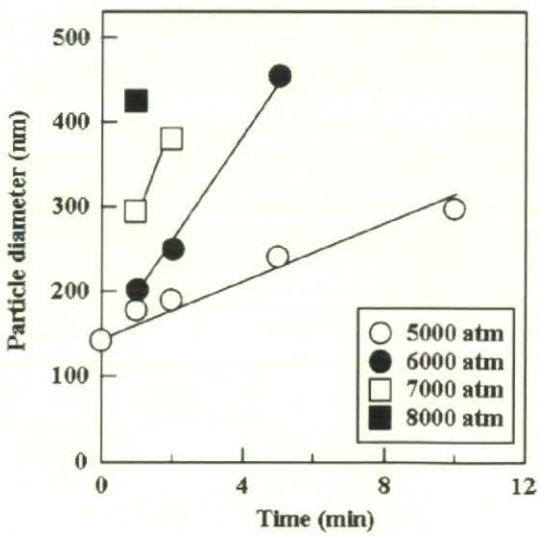

Figure 2. DLS measurements of a 10 w/v % PVA solution pressurized under various conditions.

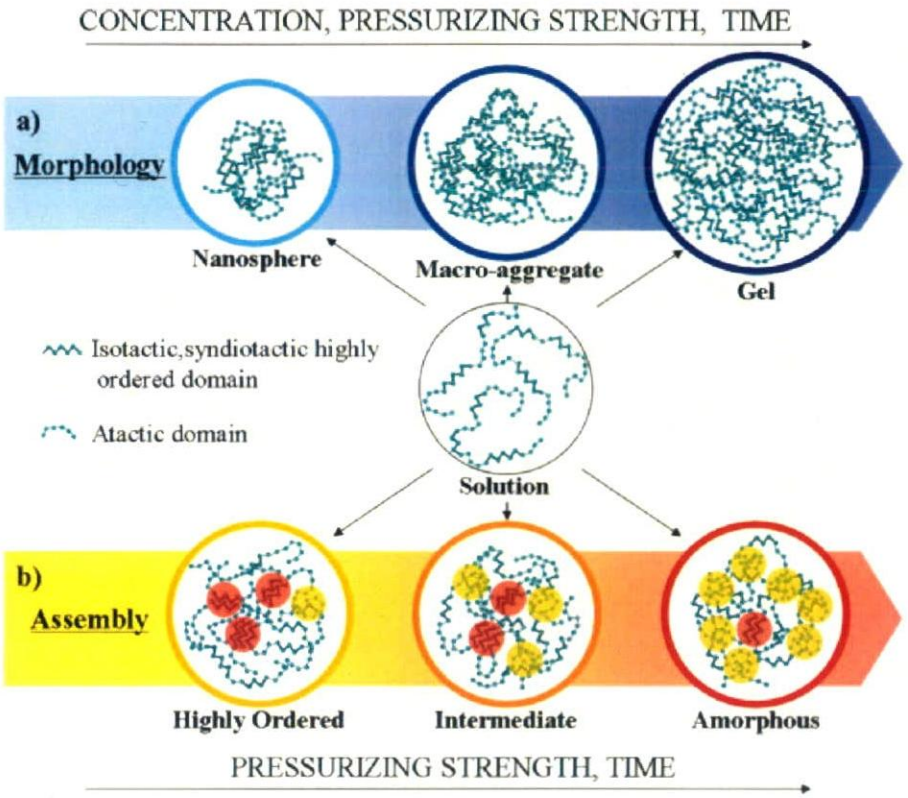

Figure 3. Illustration of the mechanism of hydrogen-bonding polymer assembly induced by ultrahigh-pressurization. (a) Effect of conditional parameters on the morphology of PVA assembly. (b) Effect of secondary structure of PVA on the formation of molecular assembly.

growth of the PVA nanoassembly under prolonged periods of pressure (Fig. 2). From these results, it is clear that the assembly of PVA at nanometer size was promoted under pressure conditions of higher pressure and a longer incubation period, and could be controlled by altering the pressurizing strength and time [Fig. 3(a)].

Characteristics of PVA Assembly Formed By Pressurization

The gelation of a PVA solution at 5, 10, 15, and 20 w/v % concentrations was also achieved by pressurization at 10,000 atm. The swelling ratio of the obtained hydrogel was determined by the starting concentration of the PVA solution, and showed a constant value for all concentrations when they were treated at 10,000 atm for more than 10 min (Fig. 4). On the other hand, the swelling ratio of the obtained hydrogel at 5 min of pressurizing time was inversely proportional to the concentration of the PVA solution (Fig. 5). This result indicates that a tight interaction

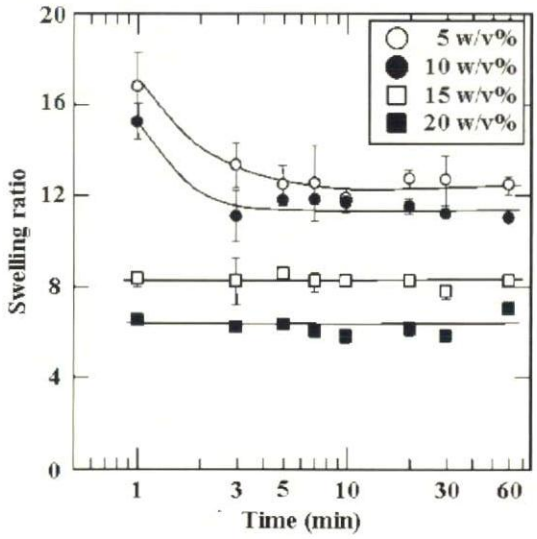

Figure 4. Effect of PVA concentration on swelling ratio of PVA hydrogel formed by pressurization at 10,000 atm for various minutes.

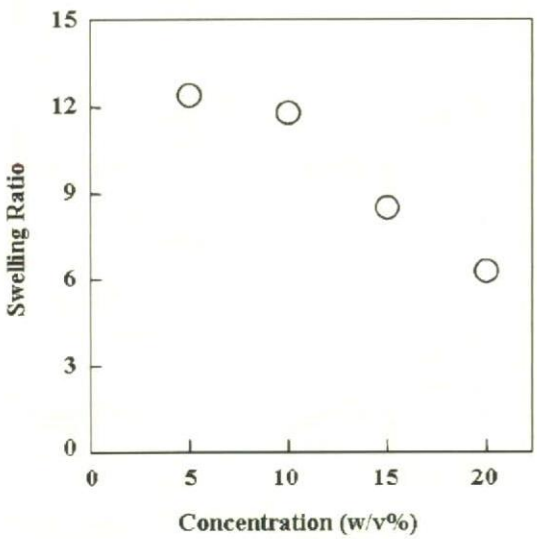

Figure 5. Swelling ratio of PVA hydrogels formed by pressurization at 10,000 atm for 5 min.

between the PVA molecules was formed with increasing the concentration of PVA solution. The interior structure of the PVA hydrogel pressurized at 10,000 atm for 10 min was observed with a SEM (Fig. 6). A mesh-like structure with pores of about 300 nm was observed for the hydrogel obtained by the pressure treatment of a 5 w/v % PVA solution. The mesh-like structures with smaller pores were formed upon increasing the PVA concentration. As the pressure treatment was carried out at 40 °C, no ice crystal was formed.[22] That is, ice crystals did not affect the mesh-like structures formed by the high-pressure process. In contrast, in the case of the freeze-thawing method, the mesh-like structures were formed by the formation of ice crystals. Therefore, a different process of formation between the two methods was suggested.

DSC analysis of the PVA hydrogels let us know the melting temperature of the associated PVA molecules. The relaxation, which occurs at a temperature between 200 and 260 °C, is caused by the melting of the crystalline domains of PVA.[23,24] The increase of intermolecular hydrogen bonding in PVA raises the melting temperature, leading to a high heat resistance.[25] The melting temperature of the PVA hydrogel

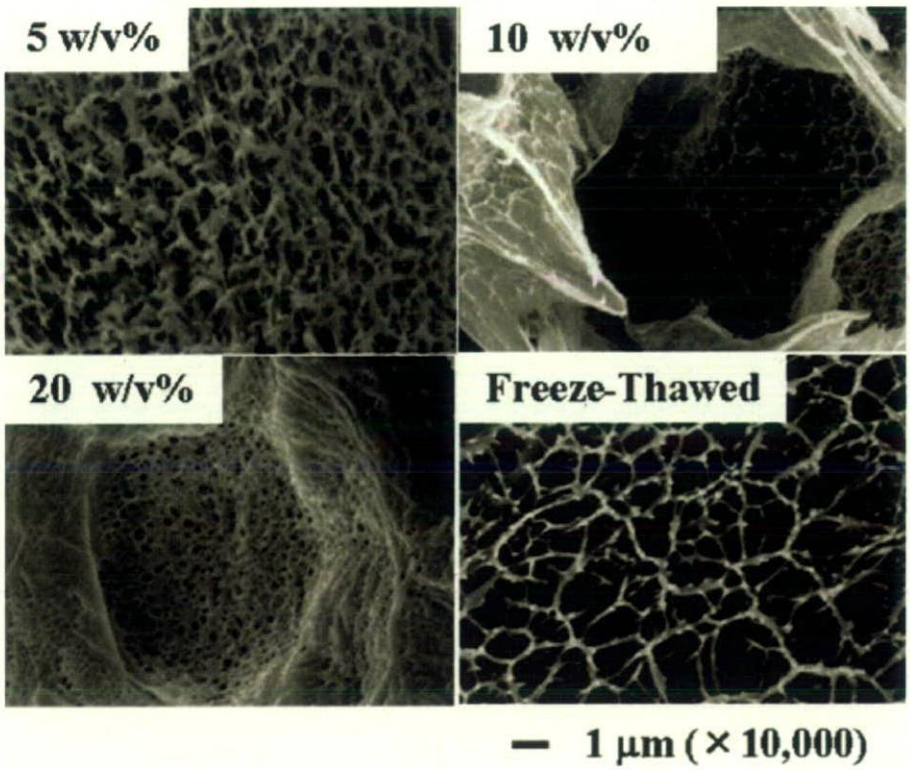

Figure 6. SEM images of PVA hydrogels of 5, 10, and 20 w/v % formed by pressurization at 10,000 atm for 10 min and 5 w/v % PVA hydrogels formed by the freeze-thawing method.

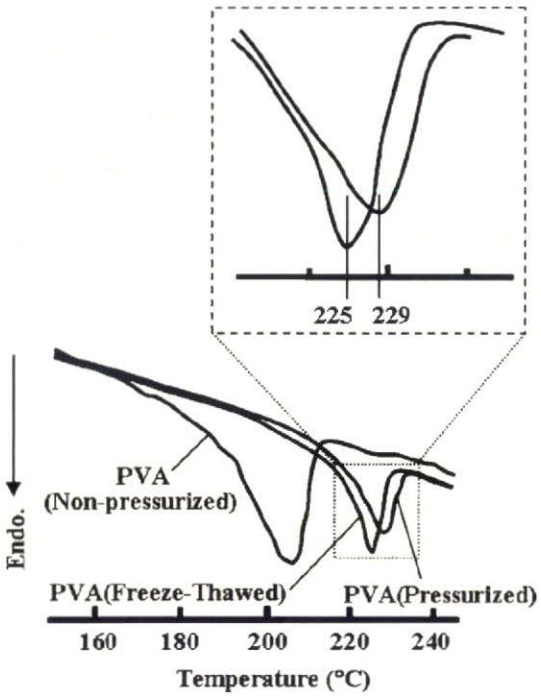

Figure 7. DSC measurements of PVA hydrogels formed by pressure treatment or the freeze-thawing method.

obtained by high-pressure process was higher than that of the hydrogel prepared by the freeze-thawing method (Fig. 7). This result indicates that high-pressure process could form stronger intermolecular interactions in PVA than the freeze-thawing method. Although we need to go into additional details about the thermodynamic stability of the PVA hydrogel obtained by high-pressure process, we have only limited information about it.

Many researchers have examined the self-organization of molecules in an aqueous environment, because the hydrogen bonds and hydrophobic interactions were able to act as a driving force for structure formation.[26–29] The formation and deformation of the hydrogen bonds in an aqueous environment can be controlled by changing the temperature and ionic concentration. The effect of the salt concentration on the high-pressure process of the PVA solution was then examined. When the NaCl concentration was increased, the PVA hydrogel was obtained even at low pressure (around 6000 atm). At over 9000 atm, stable PVA hydrogels were obtained at any salt concentration, and the swelling ratio was almost constant (Fig. 8).

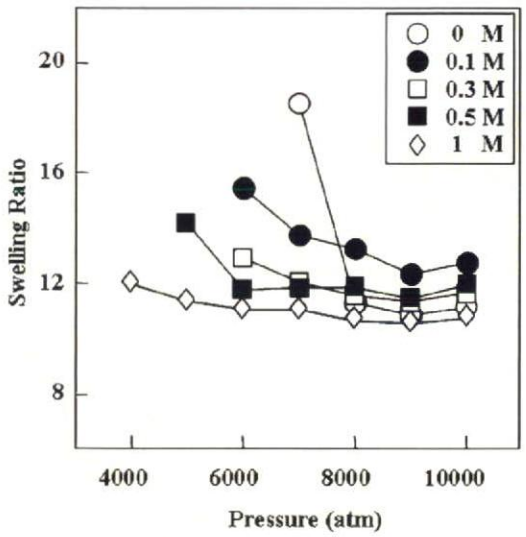

Figure 8. Swelling ratio of 10 w/v% PVA hydrogels formed by pressurization at 10,000 atm for 5 min with various salt concentrations.

When PVA solutions of less than 1 w/v % concentration were treated with pressurization at 10,000 atm, clear and turbid solutions were obtained, as well as in the case of a 10 w/v % PVA solution pressurized under low atmospheric pressure for a short time. The formation of small particles with a diameter of about 200–400 nm was confirmed from SEM observation and DLS measurements (Fig. 9). As a result, it was believed that the formation of intra/inter-molecular hydrogen bonds is the first step in the initial structural formation of PVA, and afterward the size and morphology of the structure is determined in proportion to the concentration of the solution.

The effect of the secondary (atactic, syndiotactic, and isotactic) structure of PVA molecule was observed by the ^{1}H NMR spectra analysis for the nongelled portion of the pressurized PVA solution (Table 1). Short-time pressurizing treatment at

Table 1. NMR Analysis of the Nongelled Portion of the Pressurized PVA Solution

	Tacticity		
	mm	mr	rr
PVA117HC	22.6	47.6	29.8
S-PVA	11.9	49.9	38.2
PVA117HC (20%, 6000 atm, 5 min)	21.4	49.0	29.6
PVA117HC (20%, 6000 atm, 10 min)	33.8	37.7	28.5
PVA117HC (10%, 7000 atm, 5 min)	20.9	48.0	31.1

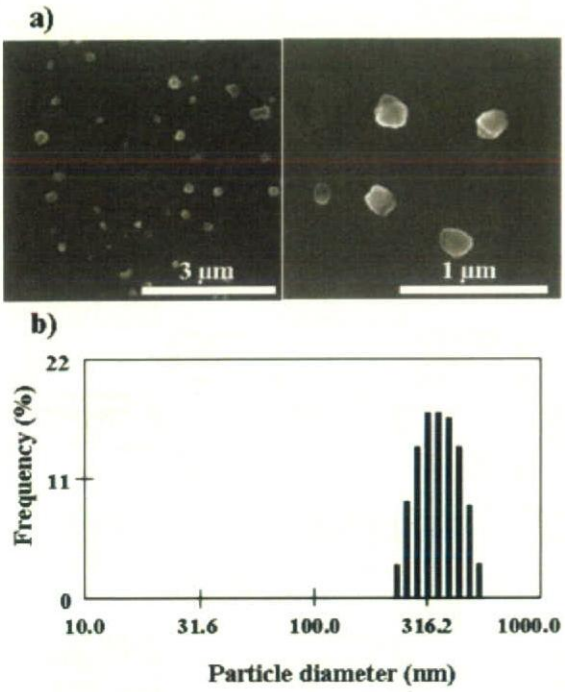

Figure 9. Small aggregates formation of PVA by pressurization. (a and b) SEM images and DLS measurements, respectively, of PVA particles formed by pressurizing a 0.5 w/v % PVA solution at 10,000 atm for 10 min.

6000 atm had no effect on the content of the secondary structure of PVA molecule, whereas after the longer treatment (10 min), the decrease of the atactic portion (mr) of PVA molecule was observed. There was no free PVA after more than 20-min treatment. These results indicated that the atactic PVA was gelled prior to other kinds of the stereostructured PVA, and after prolonged treatment all kinds of stereostructured PVA gelled. These differences of aggregation ability of each stereostructured PVA could be applicable to form the ordered structure by changing treatment time, pressure, and the content of each secondary structures of PVA [Fig. 3(b)].

The High-Ordered Structure of PVA Assembly

The assembly of PVA depended on the strength and period of pressurization and the PVA concentration. It should be noted that molecular assembly is formed through two processes induced by pressurization, which are dehydration and the subsequent formation of hydrogen bonds among inter/intra-molecules. Indeed, it is believed that under pressurized conditions, the hydration shell of the PVA molecules was disrupted, and then hydrogen bonding interactions between the hydroxyl groups of the PVA were formed. Thus, the gelation of PVA was promoted by increasing the pressure. It seems that the reaction could proceed with a long duration of pressurization even at moderate pressures. With regard to the concentration-assembly relationship, monodispersed and nanometer-scale structures were formed by intramolecular interactions under dilute conditions, whereas the macrostructure (larger than mm) was formed by the intermolecular interactions between nanometer-scaled structures which contained molecular entanglements under concentrated conditions. To construct a well-defined molecular assembly, it is necessary to optimize the primary chemical structure of the polymer molecules, and to fabricate molecules with a specific structure by exploiting various interactions. The intermolecular force maintaining the structure of the supramolecular assembly includes van der Waals force, electrostatic interactions, hydrophobic interactions, and hydrogen bonds, etc. The individual interaction energy of a hydrogen bond is small, while if it interacts along the chain direction, hydrogen bond is able to maintain a huge PVA hydrogel by assembling high-molecular weight PVA moieties. The most important factor that influences the structure formation induced by high pressure is the chain length and the secondary structure of the PVA molecule, as well as the temperature, concentration, and ionic concentration. Controlling the factors, it is expected that the ordered structures of molecular assembly can be generated. In a conventional technique, changing the concentration of the solution or a substitution of the solvent makes it difficult to change the molecular-assembly situation gradually. On the other hand, the pressuring conditions can be reversibly controlled and highly controlled operation for molecular assembly by building the interactive part, which works at a different pressure in the molecules. In the case where two or more hydrogen bonding functional groups are present, the control of a higher-order structure can be achieved by pressurizing in a stepwise fashion. We assumed that the secondary structure of PVA is one of the most possible candidates for the factors for obtaining the ordered molecular assembling structure. It is expected that such technology can be applied to build a structure by the manipulating molecular interactions to develop

novel structure in aqueous solution, leading to new science and technology.

CONCLUSIONS

The PVA assembly was simply obtained through an ultrahigh-pressure process. The morphology of PVA assembly depended on the strength and period of pressurization, the PVA concentration, the PVA chain length, and the PVA secondary structure. Under the ultrahigh-pressure, molecular assembly is formed through two processes, which are dehydration and the subsequent formation of hydrogen bonds among inter/intramolecules. Thus, the ultrahigh-pressure process can manipulate molecular interactions. Therefore, it is expected that the novel high-ordered structures based on molecular assembly can be generated by controlling various factors in an ultrahigh-pressure process.

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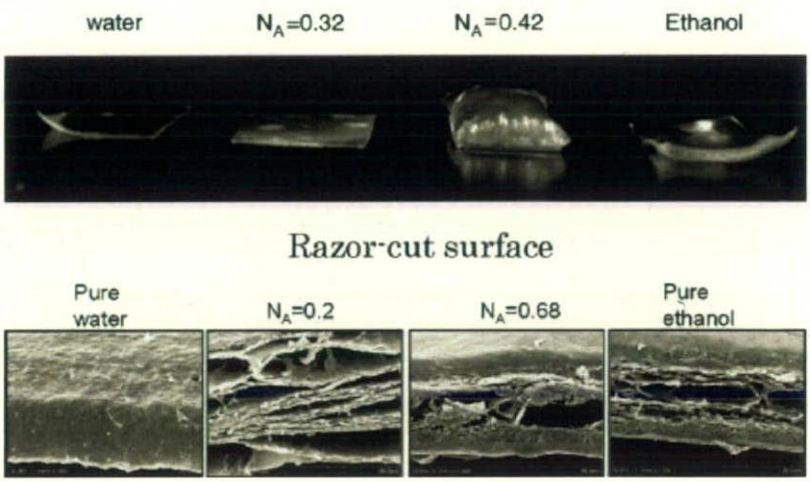
Controlling Coupling Reaction of EDC and NHS for Preparation of Collagen Gels Using Ethanol/Water Co-Solvents

Kwangwoo Nam, Tsuyoshi Kimura, Akio Kishida*

To control the crosslinking rate of the collagen gel, ethanol/water co-solvent was adopted for the reaction solvent for the collagen microfibril crosslinking. Collagen gel was prepared by using EDC and NHS as coupling agents. Ethanol did not denaturate the helical structure of the collagen and prevented the hydrolysis of EDC, but showed the protonation of carboxylate anions. In order to control the intra- and interhelical crosslink of the collagen triple helix, variations of the mole ratio of carboxyl group/EDC/NHS, and of the ethanol mole concentration were investigated. Increase in the EDC ratio against the carboxyl group increased the crosslinking rate. Furthermore, an increase in the ethanol mole concentration resulted in an increase of the crosslinking rate until ethanol mole concentration was 0.12, but showed gradual decrease as the ethanol mole concentration was further increased. This is because the adsorption of solvent by the collagen gel, protonation of carboxylate anion, and hydrolysis of EDC is at its most optimum condition for the coupling reaction when the ethanol mole concentration is 0.12. The re-crosslinking of the collagen gel showed an increase in the crosslinking rate, but did not show further increase when the coupling reaction was executed for the third time. This implied that the highest possible crosslinking rate for the intra- and interhelical is approximately 60% when EDC/NHS is used.



Introduction

The construction of an extracellular matrix (ECM) using natural products has been performed by many researchers worldwide. Based on the fact that an ECM is mainly composed of collagen and elastin, many researchers have

K. Nam, T. Kimura, A. Kishida
Division of Biofunctional Molecules, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan
Fax: 03-5280-8029; E-mail: kishida.fm@tmd.ac.jp

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attempted to prepare a collagen- or elastin-based material to construct an ECM. Ever since Weinberg and Bell succeeded in preparing a blood vessel using collagen,[1] diverse approaches using collagen gel to prepare an ECM had been executed. However, the critical aspect in using collagen gel is that its mechanical strength is too small and easily deforms its triple-helix structure into a random coil structure when heated. The low mechanical strength and easy deformability make collagen shrink easily due to external stimuli. These aspects make it difficult to use collagen as an ECM. The use of crosslinkers to overcome these problems was investigated and is well reviewed by Khor.[2] By crosslinking collagen triple-helices, it is possible to maintain its mechanical strength and suppress any deformation caused by external stimuli. However, it is very important to consider biological responses in the designing stage of a crosslinking process because of the possibilities of severe problems such as toxicity, inflammatory response or the alteration of protein structure.

A crosslinking method using 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in aqueous condition is a one of the best methods to produce a non-toxic collagen product. This reaction mixture induces the formation of an amide bond by activation of the side chain carboxylic acid groups of aspartic and glutamic acid residues, followed by aminolyis of the *o*-isoacylurea intermediates by the ε-amino groups of (hydroxy-)lysine residues, forming intra- and interhelical crosslinks.[3–5] A coupling reaction that involves EDC depends on the amount of EDC and on the EDC/NHS ratio.[4–6] A higher EDC and NHS mole ratio against the carboxylic groups increases the coupling reaction rate. The pH of the solvent for the coupling reaction should be higher than the pK_a value, which is 5.8 for collagen. This is because the carboxylate anions otherwise exhibit a higher coupling rate than that exhibited by the carboxyl groups.[6] The coupling reaction using EDC is one of the most widely used crosslinking methods in the biomaterials field; however, it is regarded as an inappropriate method, especially in tissue engineering, owing to its extremely low coupling efficiency. This is because EDC tends to hydrolyze rather rapidly under aqueous conditions.[3–7] The use of NHS to suppress the hydrolysis does not function to the desired extent. Furthermore, since collagen consists of triple helices, the efficiency of the coupling reaction is lower than that of crosslinkers such as diol-related crosslinkers or glutaraldehyde because the only possible reactions are the intra- and interhelical coupling reactions. Hence, the question of whether it is possible to control the coupling reaction rate of EDC for collagen crosslinking was brought up.

Our research group attempted to control the coupling reaction of EDC/NHS using the collagen gel. We found out that in order to obtain a crosslinked collagen gel that is mechanically tough and possesses a low swelling ratio, collagen should be crosslinked under neutral or alkaline pH conditions with the EDC/NHS/carboxylate anions in a ratio of 10:10:1.[4] The swelling ratio in pH 7.4 was less than 150%, which is approximately 1/5 of that of the uncrosslinked collagen gel. It was shown that no denaturation of the triple helix had occurred. The elastic modulus increased to approximately 4.8 times that of the uncrosslinked collagen gel. However, when we investigated the free amine group contents, the lowest value of that we could obtain was approximately 60%. Glutaraldehyde crosslinking on the same collagen gel revealed that the free amine group content was less than 15% and the diol-related crosslinker exhibited an approximate free amine group content of 30%.[7] We concluded that this is the lowest possible coupling reaction rate for the collagen microfibrils under aqueous conditions. Thereafter, we started to search for new conditions for collagen crosslinking using EDC and NHS. In this study, we attempted to control the EDC/NHS coupling reaction rate by making the reaction environment highly hydrophobic. To achieve the more hydrophobic environment, we used ethanol, which is miscible with water. Ethanol/water mixed solvents were prepared in different mole concentrations to control the hydrophobicity of the solvent. There are a number of research papers on the reaction of EDC/NHS with collagen in ethanol, but it is not completely clear as to how the EDC and NHS coupling reaction would be affected when the alcohol percentage in aqueous conditions changes; hence, different ethanol concentrations are being used without characterization of the coupling rate.[8–11]

Experimental Part

Preparation of Collagen Gel

The preparation of the collagen film was performed by the same method as that reported previously.[5,7] A 0.5 wt.-% solution of collagen type I (I-AC, KOKEN, Tokyo, Japan) was concentrated into a 2 wt.-% collagen type I solution and used for the film preparation. The collagen solution was dropped onto a polyethylene film and dried at room temperature. A transparent film with a thickness of 56 ± 3 μm was obtained. The films were stored in a dry environment.

To investigate the effect of the solvent, the collagen film was immersed into an ethanol/water mixed solvent containing EDC and NHS (both from Kanto Chemicals, Tokyo, Japan). Each chemical was added in the mole ratio of EDC/NHS/collagen-carboxylic acid group = 10:10:1. The ethanol mole concentration (N_A) was changed from 0 to 1 [ethanol/water ratio from 10:0 to 0:10 (v/v)]. The crosslinking procedure was allowed to continue for 24 h at 4 C to produce a crosslinked gel (EN gel). After 24 h, the reaction was terminated by removing the gel from the solution. The gel was then washed with distilled water for 3 d in order to remove any unreacted chemicals from the collagen gel. For the

re-crosslinking process, the same procedure as above was repeated using water, $N_A \approx 0.12$, $N_A \approx 0.42$ and 100% ethanol as the reaction solvent. Crosslinking of the collagen gel to glutaraldehyde was performed by using a 0.5 wt.-% glutaraldehyde solution (Merck, Darmstadt, Germany) in a phosphate buffer solution (PBS).[12] The collagen film was immersed in the glutaraldehyde/PBS solution and was crosslinked for 3 h at room temperature. After crosslinking, the sample was first rinsed under running tap water for 30 min and then in 4 M NaCl for 2 h. In order to eliminate NaCl, the sample was rinsed with distilled water for 1 d to yield a glutaraldehyde-crosslinked collagen gel. The 1,4-butanediol diglycidyl ether (BDDGE)-crosslinked collagen was prepared by immersing a collagen film in a 4% BDDGE/PBS solution and reacting for 5d.[13] The BDDGE-crosslinked collagen was left under running tap water for 15 min to wash off the unreacted BDDGE. The washing process was repeated several times. The glutaraldehyde-crosslinked collagen gel and the BDDGE-crosslinked collagen gel were used for the characterization of the free amine group content.

Characterization of the Collagen Gel

A solubility test was performed in the ethanol/water mixed solvents. The collagen films (3–4 mg) and collagen chunks obtained from lyophilization (7–10 mg) were immersed in ethanol/water mixed solvents. The collagen solutions were left at room temperature until complete dissolution occurred. The triple-helix structure was characterized using a circular dichroism (CD) spectrometer (J-720W, Jasco, Tokyo, Japan). Collagen solution was prepared at a concentration of 1×10^{-7} M and characterized 5 times for each sample to obtain the average spectra. Surface analysis was performed by scanning electron microscopy (SEM, SM-200, Topcon, Tokyo, Japan). The same solubility test was repeated using the collagen film. The diffusion coefficient D was calculated using a collagen gel that was prepared in a 2-(*N*-morpholino)ethansulfonate (MES) buffer. The collagen gels were immersed in the ethanol/water mixed solvents at pH 9.0. The gels were then removed at 10, 60, 120, 240, 360, 1 440, and 4 320 min (3 d) and the adsorbed amounts of the solvent were measured. The following equation was used for the calculation of D:

$$M_t/M_\infty = 4(Dt/\pi l^2)^{1/2}, \quad (1)$$

where M_t and M_∞ are the amounts of the solvent adsorbed at time t and at infinity, respectively and l is the thickness of the collagen gel.[14,15]

The primary amine group concentrations in the tissue samples were determined using a colorimetric assay.[16,17] From each sample a 2–4 mg specimen was prepared. These samples were immersed in a 4 wt.-% aqueous $NaHCO_3$ solution (Kanto Chemicals, Tokyo, Japan) and a 0.5 wt.-% aqueous solution of 2,4,6-trinitrobenzene sulfonic acid (TNBS; Wako chemicals, Osaka, Japan) was added. The reaction was allowed to continue for 2 h at 40 °C, after which the samples were rinsed in saline solution using a vortex mixer to remove the unreacted TNBS. The samples were freeze-dried overnight, after which the dry mass was determined. The dry samples were immersed in 2 mL of 6 M aqueous HCl until fully dissolved. The obtained solution was then diluted with distilled water (8 ml) and the absorbance was measured at 345 nm (V-560, Jasco, Tokyo, Japan). The concentration of the reacted amine groups was calculated using the following equation:[16,17]

$$[NH_2] = (A \cdot V)/(\varepsilon \cdot l \cdot m) \quad (2)$$

where $[NH_2]$ denotes the reacted amine group content [in mol/g of collagen gel]; ε, the molar absorption coefficient of trinitrophenyl lysine (1.46×10^4 $l \cdot mol^{-1} \cdot cm^{-1}$); A, the absorbance; V, the volume of the solution [mL]; l, the path length [cm]; and m, the weight of the sample [mg]. The free amine group contents were calculated by assuming that the uncrosslinked collagen gel has 100% free amine groups.[7,8] The experiment was repeated five times and the average along with the standard deviation was calculated.

All the experiments were repeated at least thrice and the values were expressed as mean ± standard deviation. In several figures, the error bars are not visible because they are included in the plot. A statistical analysis was performed using the student's t test with the significance level set at $p < 0.05$.

Results and Discussion

We started by setting up three hypotheses: 1) ethanol does not denaturate the triple helix, 2) ethanol prevents the hydrolysis of EDC, and 3) the carboxyl groups are reactive with EDC in ethanol. These three hypotheses are important in the aspect that the failure of one hypothesis implies that the collagen crosslinking is meaningless. Hence, the experiment was conducted by proving the hypotheses one by one. We first started with the characterization of the triple helix of the collagen. The exposure of the collagen triple-helices to ethanol induces hydrophobic interactions, which may lead to a change in the conformation of the collagen microfibrils. Using a CD spectrometer, we observed the conformation structure of collagen in the range of $N_A \approx 0$–0.42 (ethanol/water = 0/10–7/3, v/v). The increase in ethanol concentration against water did not bring about any distinguishable change in the triple helical structure (Figure 1). The positive band and the cross-band seen in the CD spectra were the same for all the tested samples ($N_A \approx 0$–0.42). The negative band exhibited a slight red-shift as the ethanol concentration was increased. However, no signs of denaturation, such as a decrease in the peak intensity of positive and negative band, were detected.[18,19] Hence, it is assumed that ethanol up to $N_A \approx 0.42$ does not change the triple helices into random coils.[20] The main forces that hold the helical structure of collagen are hydrogen bonds, electrostatic interactions, and hydrophobic interactions. In water, the hydrogen bonds and electrostatic interactions within collagen contribute to the stabilization of the helices, but they are not the dominant factors.[20] The structure of collagen depends on the concentration of the alcohols. This

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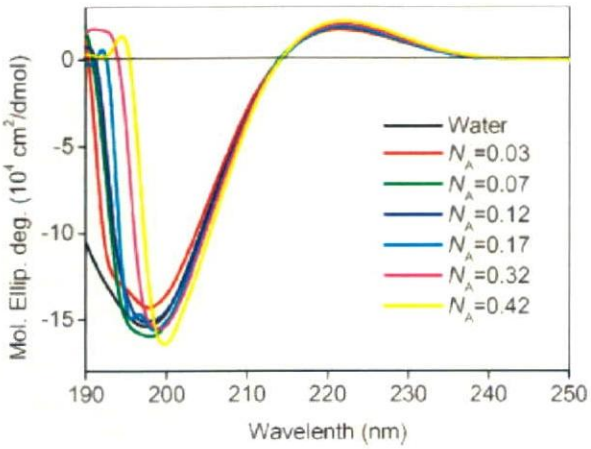

Figure 1. CD spectra of the collagen microfibrils under various ethanol mole concentrations.

is because an increase in the hydrophobic interactions between the solvent and collagen stabilized the structure of collagen.[21] The hydrophobic interactions between the non-polar amino acid side chains are also very important factors that contribute to the stabilization of the helices. Exposure of the non-polar amino acid side chains to the outer side would induce hydrophobic interactions, which were not observed under aqueous conditions. This causes a hydrophobic shielding effect.[22] However, it is generally assumed that this tendency is strongly influenced by the type of alcohol used. Thus, polyhydric alcohols such as sorbitol or glycerol favour the native structure, while monohydric alcohols enhance the native structure.[23] In the case of ethanol, the secondary and tertiary structures of collagen would be affected.[22,24] As result, it is assumed that the transformation 'triple helix → random coil' does not occur, and the use of ethanol for the amide coupling reaction for collagen crosslinking is preferable. The triple-helix structure at $N_A > 0.55$ was measured indirectly. That is, since the random coil is not reconverted to the triple-helix structure,[22] we resolubilized collagen in water and observed the CD spectra and concluded that the collagen structure would remain a triple helix even at extremely high ethanol mole concentrations.

However, it should be noted that the use of ethanol is not a solution for the control of the coupling reaction. The surface of collagen is too hydrophobic and rigid, in which the fibrillar structure disappears. The solubility test showed that the ethanol mole concentration should be at least 0.42 to dissolve collagen. The same phenomenon was observed for the collagen film. The collagen film, which is un-crosslinked, could be dissolved at $N_A \approx 0.42$, but would remain undissolved in higher hydrophobic conditions. Expectedly, the time required for complete dissolution was different, where high-hydrophobic conditions delayed the dissolution time. Figure 2 shows the morphology of collagen microfibrils observed by SEM. It is seen that the microfibril structures disappear as the hydrophobicity increases. The disappearance of the fibrillar structure decreases the absorptivity of the solvent. This suggests that for the collagen film, the adsorption of ethanol by the collagen gel would be extremely low. To prove this, we have calculated the diffusion coefficients D for various mole concentrations of ethanol, as shown in Figure 3, using the collagen gel crosslinked with EDC/NHS in a MES buffer that was prepared by the method reported previously.[5] This shows that the D of the solvent decreases rapidly when $N_A \geq 0.55$ (ethanol/water = 8/2,

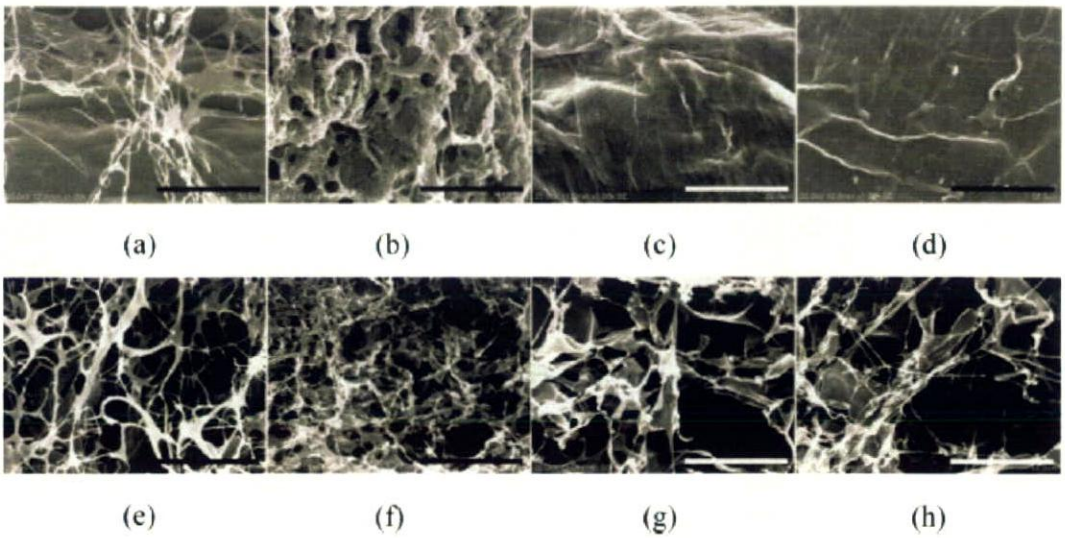

Figure 2. Morphology of collagens after immersing in ethanol/water mixed solvents of different concentrations. (a) Water, (b) $N_A \approx 0.07$, (c) $N_A \approx 0.17$, (d) $N_A \approx 0.32$, (e) $N_A \approx 0.42$, (f) $N_A \approx 0.55$, (g) $N_A \approx 0.73$, and (h) ethanol. Single bar indicates 50 μm.

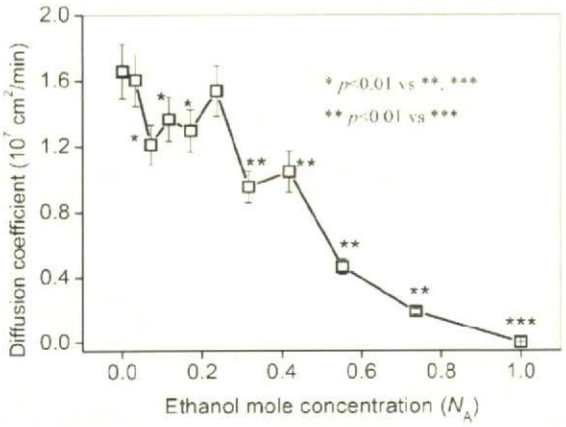

Figure 3. Change in the diffusion coefficient of ethanol in collagen gel according to ethanol mole concentrations.

v/v); furthermore, the D value of pure ethanol (1.2×10^{-10} cm$^2 \cdot$ min^{-1}) is approximately 1400 times lower than that of pure water. This directly affects the crosslinking ability. The solvent adsorption ability in pure ethanol and at $N_A \approx 0.74$ (ethanol/water = 9/1, v/v) is about 50% of that of pure water and 80% at $N_A \approx 0.55$ after 24 h of solvent adsorption. This implies that ethanol could not completely reach the interior of the collagen gel throughout the crosslinking procedure.

Using EDC and NHS, we obtained crosslinked collagen gels under various ethanol concentrations (Figure 4). When EDC and NHS are used for the crosslinking process, the lowest value of the free amine group content was approximately 45% (60% when crosslinked in MES buffer). This can be achieved when the crosslinking was executed for 24 h at $N_A \approx 0.07$–0.17 (ethanol/water = 2/8–4/6, v/v) with 51 mmol of EDC. This range is assumed to be the most proficient range for the coupling reaction, where the suppression of hydrolysis and fast solvent absorption has occurred. The addition of ethanol is thought to have prevented the hydrolysis of EDC. On the other hand, when

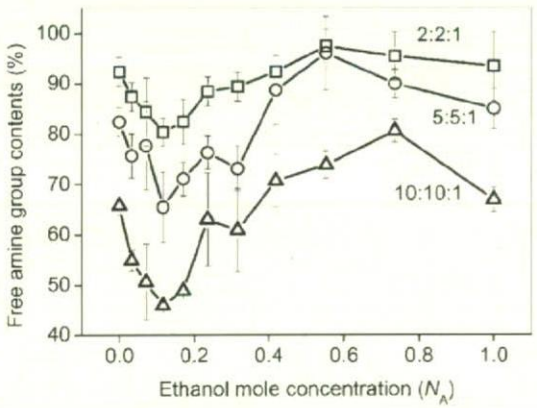

Figure 4. Change in the free amine group contents of collagen gel according to ethanol mole concentrations.

$N_A \approx 0.24$ (ethanol/water = 5/5, v/v), the free amine group content increases again, and from $N_A \approx 0.42$ and above, the free amine group content increases to higher than that of pure water. This is because of the decrease in the number of carboxyl groups reacting with EDC.[25,26] The reactivity of the carboxyl groups decreases as the ethanol concentration increases because EDC reacts with the carboxylate anions. The increase in the number of neutral carboxyl groups would lead to relatively low *O*-isoacylurea formation.[6] Furthermore, when $N_A \geq 0.42$, the crosslinking is assumed to be mainly concentrated on the surface of the collagen gel. The decrease in D causes heterogeneous coupling reactions in the collagen gel. That is, the partly crosslinked network of the collagen gel could be mainly located on the surface of the gel. This can be confirmed when the collagen gels prepared at $N_A \geq 0.42$ are placed in pure water. The sudden change in the environment causes the gel to adsorb a large amount of water, which makes the uncrosslinked collagen microfibrils dissolve and expand to the maximum extent by an increase in the free energy. The expansion of the collagen microfibrils is obstructed by the crosslinked part, which is mainly located on the surface. For the collagen gel prepared at $N_A \approx 0.42$, D is approximately the same as that of the gel prepared at $N_A \approx 0.32$, but it is thought that the protonation of the carboxyl groups prevents the formation of *O*-isoacylurea. The reactivity between the carboxyl groups and D alters the formation of the collagen gel. When the morphology of the razor-cut surface was observed, the monolithic morphology of the collagen gel was found to form a layered structure as the hydrophobicity increased, which eventually collapses. The collapse of the inner part of the collagen gel is due to the dissolution of the uncrosslinked collagen microfibrils. This implies that the crosslinking of the collagen gel would start from the surface and then occur inside the collagen gel. Furthermore, it is possible to crosslink only the surface of the collagen gel to obtain a phase-separated collagen gel when the ethanol concentration is controlled.

An extended reaction time under high-hydrophobic conditions ($N_A \geq 0.42$) did not cause any significant difference in the free amine group content. The crosslinking rate is much higher after 24 h, as compared to 4 h; however, no significant change is observed after 48 h. When crosslinking was performed in MES buffer, we observed a decrease in the free amine group content;[7] however, in the case of ethanol, the formation of the *O*-isoacylurea does not occur due to the slow adsorption and protonation of the carboxyl groups.

Is it possible to obtain a collagen gel with a smaller number of free amine groups? To answer this question, we have re-crosslinked the collagen gel by repeating the same procedure (Figure 5). The activation by EDC can be triggered when EDC is introduced into the reaction solvent.[4] We

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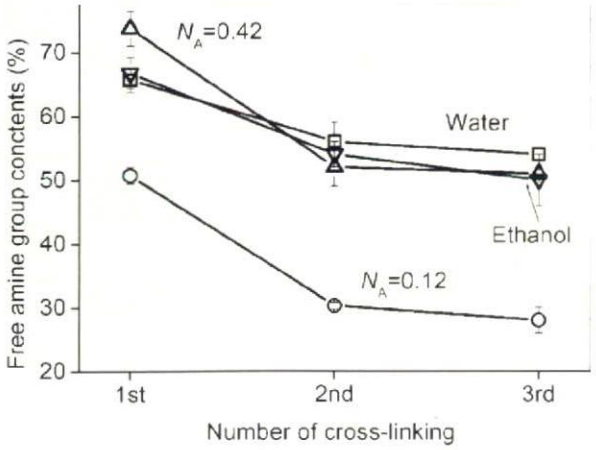

Figure 5. Change in the free amine group content of collagen gel by the re-crosslinking procedure in different solvents.

have proved in our previous report that the carboxyl groups can be activated at any point of time during the course of the reaction.[6,7] Thus, by re-crosslinking the collagen gel, we attempted to evaluate the highest coupling rate possible using this process. The re-crosslinking was possible and the least value of the free amine group content was 30% ($N_A \approx 0.12$). This value is still high as compared with the glutaraldehyde-crosslinked collagen gel (≈12% using the same collagen gel) and the BDDGE-crosslinked collagen (≈25% using the same collagen gel). This is thought to be the lowest limit of the EDC/NHS crosslinker. Unlike glutaraldehyde and BDDGE, which can interconnect the microfibrils of the collagen, EDC/NHS can only induce intra- and interhelical crosslinks. It is difficult to assume that the microfibrils are crosslinked via the EDC/NHS crosslinker due to distal problem. Hence, it is not possible to achieve a free amine group content that is lower than ≈30%. The crosslinking may still occur when a different crosslinker or a polymer is added to this collagen gel.

Conclusion

We have proposed a new method for controlling the coupling reaction rate using EDC and NHS for collagen crosslinking. The collagen triple-helix was stable in ethanol/water mixed solvent, but the properties of the collagen gel prepared in the above solvent could be altered by the ethanol mole concentration. The highest reaction rate was achieved at $N_A \approx 0.07$–0.17 with 51 mmol of EDC in 24 h. This is the optimum concentration range that balances the reactivity of EDC and the formation of carboxyl groups. We also discovered that the coupling reaction begins from the surface of the collagen gel. The coupling reaction was limited to the surface of the collagen when $N_A > 0.55$; this was because of the slow penetration of EDC and NHS caused by the high-ethanol environment and the decrease in the number of carboxylate anions. It is thought that the same procedure could be repeated not only in collagen but also in collagen-based materials such as body tissue and proteins.

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Keywords: collagen gel; crosslinking; EDC; ethanol

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Cell removal with supercritical carbon dioxide for acellular artificial tissue

K. Sawada,[1]* D. Terada,[2] T. Yamaoka,[3] S. Kitamura[3] and T. Fujisato[2]

[1]Department of Integrated Life, Osaka-Seikei College, 3-10-62, Aikawa Higashiyodogawa-ku, Osaka, 533-0007, Japan
[2]Department of Biomedical Engineering, Osaka Institute of Technology, 5-16-1 Omiya, Asahi-ku, Osaka, 535-8585, Japan
[3]National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

Abstract

BACKGROUND: The objective of this work was to decellularize artificial tissue without using surfactant solutions. For this purpose, supercritical carbon dioxide was used as the extraction medium.

RESULTS: Supercritical carbon dioxide containing a small amount of entrainer was a suitable medium to extract both cell nuclei and cell membranes from artificial tissue. Under gentle extraction conditions (15 MPa, 37 °C), cell nuclei were satisfactorily extracted from tissue within 1 h. In contrast, the efficiency of phospholipid removal depended strongly on the transfer rate of carbon dioxide in the interior of the tissue. Mechanical strength of tissue was not decreased even with prolonged treatment.

CONCLUSION: Acellular artificial tissues could be prepared quickly by treatment with a carbon dioxide/entrainer system. The prepared acellular tissue could be obtained in absolutely dry condition. This is advantageous from the viewpoint of long-term preservation without putrefaction and contamination.

Keywords: supercritical carbon dioxide; extraction; decellularization; aorta

INTRODUCTION

In recent years, the regeneration of diseased heart valves by implantation of artificial or animal alternatives has been investigated extensively.[1–5] To accomplish complete tissue regeneration, several problems need to be overcome. For example, acute rejection of the implanted graft must be prevented. The graft must be gradually degraded without losing apparent mechanical strength, and gradually replaced with regenerated recipient tissue after implantation. Furthermore, the recipient's cells should penetrate effectively into the graft. Heart valve replacements are currently performed using synthetic biodegradable polymers such as polylactic acid and polyglycolic acid.[6–9] However, complete reproduction of the heart valves using these synthetic polymers is very difficult because of the complicated structure of the valves. In addition, the mechanical properties of the synthetic polymers, which are used to make long-standing implants, are not equivalent to those of native tissues.

In contrast, replacements of diseased heart valves with healthy valves from animals have been reported recently; the replaced heart valve is markedly more durable than valves made from synthetic polymers. However, strict evaluation of safety for the recipient is necessary. The most important factor to be evaluated is the extent of removal of donor cells, because residual donor cells are the major cause of rejection. Various decellularization methods have been proposed to remove these cells.[10–13] An example of these methods is the use of surfactants, such as SDS and TritonX-100.[14–20] SDS and Triton X-100, which are potent reducers of surface tension, show strong detergent properties and consequently the cells in the tissue appear to be effectively washed out. However, complete removal of toxic surfactants from the tissue is difficult even after repeated rinsing, since they show high affinity to the extracellular matrix. In addition, cross-linking of tissue with a suitable aldehyde is generally performed after surfactant treatment to maintain the mechanical properties of the tissue. However, this process is thought to be related to calcification of the tissue after implantation. Alternative decellularization methods that do not involve the use of chemicals must be effective in overcoming these problems.

In a previous study, a decellularization process for porcine tissue was developed using ultrahigh pressure

* Correspondence to: K. Sawada, Department of Integrated Life, Osaka-Seikei College, 3-10-62, Aikawa Higashiyodogawa-ku, Osaka, 533-0007, Japan
E-mail: sawada-k@osaka-seikei.ac.jp
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in aqueous solutions.[21] The use of ultrahigh pressure and subsequent rinsing in aqueous solution showed excellent effectiveness in removing the cells without the use of surfactants or other chemicals. In addition, inactivation of retrovirus was also attained without losing the mechanical strength of the tissue. However, phospholipids were not removed from the cell membrane, although the cell nuclei were completely removed. To remove the remaining phospholipid from the tissue, additional rinsing in alcohol for 3 days was necessary. Furthermore, more than 2 weeks was necessary to complete this process. Because of the prolonged treatment and the need for several steps when this method is used, a simpler decellularization procedure is desirable.

In this study, a novel method for the decellularization of tissue that did not require a long period for completion and did not involve multiple steps was investigated. The process used the supercritical fluid extraction technique. Supercritical fluids are used in a variety of industrial fields. One of the attractive characteristics of supercritical fluids is their transport coefficients. For example, the diffusion coefficient of a supercritical fluid is intermediate between those of gases and liquids. On the other hand, the viscosity of a supercritical fluid is equivalent to that of a gas. Consequently, a supercritical fluid has a high transfer rate and high permeability. Furthermore, these values can be changed continuously by varying the temperature and pressure. Thus far, supercritical fluids have been applied in the selective extraction of valuable materials in fields such as pharmacy and food science.[22,23]

The strategy for the decellularization of tissue was to use supercritical fluid extraction of the cells by varying the permittivity of the medium. This method has the potential to extract cells effectively with a single extraction medium by controlling the operating pressure. In this study, carbon dioxide was selected as the extraction medium and porcine aorta as the tissue. Because carbon dioxide has moderate critical conditions ($T_C = 32\,^{\circ}C$, $P_C = 7.38\,MPa$), the mechanical properties of the tissue are unlikely to be lost during treatment under supercritical conditions. In addition, carbon dioxide in the tissue will return to the gaseous state after treatment and will naturally diffuse toward the outside of the tissue. As a result, there is no possibility of chemicals remaining in the tissue. Thus, decellularized tissue prepared by this method will be safe for the recipient after implantation.

This study was a fundamental investigation to collect basic information for future application of the process to other organs, such as heart valves and trachea.

EXPERIMENTAL

Materials

Porcine aorta was isolated from a Clawn miniature pig obtained from Japan Faram Co. Ltd. Before treatment of the aorta with supercritical carbon dioxide, moisture on its surface was removed with filter paper. In all experiments, the aorta was cut into 1 g pieces. Pure grade carbon dioxide (>99.9%) was used, purchased from Sumitomo Seika Chemicals Co. Ltd, Japan. If necessary, ethanol was used as an entrainer. Ethanol (SIGMA-ALDRICH Japan K.K., Japan) was of reagent grade and was used after drying with a 3A molecular sieve.

Procedure

Decellularization of porcine aorta was examined using a high-pressure reaction apparatus. Figure 1 is a schematic diagram of the apparatus. The main components of the apparatus include a pressurizing pump, stainless steel vessel, and backpressure regulator. The pressurizing pump (SCF-Get; Jasco Corporation, Japan) supported the flow of carbon dioxide at the desired rate. The stainless steel vessel had a total volume of 50 cm^3 and a diameter of 3 cm. The vessel was placed in a water bath to control the treatment temperature. The contents of the vessel could be stirred with a Teflon-coated bar driven by an outside magnet. The backpressure regulator (Jasco Corporation) was a computer-controlled machine and was able to release carbon dioxide at the desired flow rate for the desired period. In this study, the releasing pressure was strictly controlled to evaluate the effect of the mass transfer rate of the fluid.

Liquid carbon dioxide from a cylinder was compressed with a pressurizing pump and made to flow into the reaction vessel until the pressure reached the desired value. When an entrainer was needed, excess ethanol (15 mL) was preloaded in the bottom of the vessel. Under the supercritical condition, the ethanol at the bottom of the vessel will dissolve in the upper phase until the carbon dioxide is saturated with ethanol. In all experiments, the aorta was fixed to the upper side of the vessel to prevent direct contact with ethanol. Consequently, in the supercritical condition, the aorta would be surrounded with supercritical carbon dioxide alone or with a mixture of supercritical carbon dioxide and ethanol fluid. Unless otherwise noted, the operating temperature was fixed at 37 °C.

Decellularization was evaluated by hematoxylin–eosin (HE) staining and quantitative analysis of phospholipids. Phospholipid was assayed using the combined enzymatic method reported by Takayama

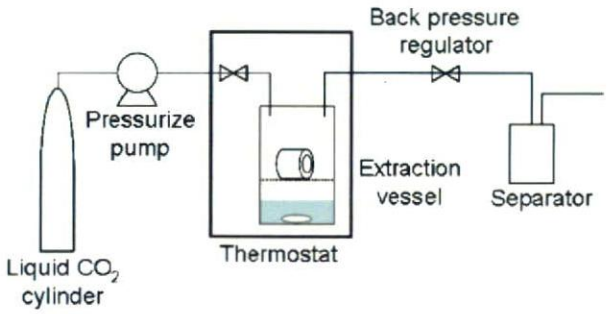

Figure 1. Schematic diagram of the experimental apparatus.

et al.[24] This evaluation method detects phospholipid selectively. To evaluate the mechanical strength of the porcine aorta, tensile strength was evaluated before and after treatment using a Tensilon RTC-1150A (Orientec Co. Ltd, Japan) tensile tester.

RESULTS AND DISCUSSION

Evaluation of decellularization

For evaluating the decellularization of tissues,[25–27] the following two factors are generally recognized as important for the safety of the recipient. The first is the evaluation of the degree of removal of cell nuclei. Because residual donor cell nuclei cause serious rejection, they must be removed as completely as possible. The second factor that should be evaluated is the extent of removal of phospholipid. Phospholipid is the main ingredient of cell membranes and the nuclear membrane. The residue of donor phospholipid is thought to cause gradual deposition of calcium phosphate. As a result, calcification of the tissue may occur. To confirm the decellularization of tissue in this study, residual cell nuclei were evaluated by tissue staining and residual phospholipid by chemical analysis.

Figure 2 summarizes HE staining of porcine aorta treated with supercritical carbon dioxide. A preliminary study confirmed that the degree of cell removal evaluated by HE staining completely agreed with the results obtained by spectrophotometric analysis of DNA. As shown in Fig. 2, the aorta that had been treated with supercritical carbon dioxide only (Fig. 2(b)) was microscopically similar to the native tissue (Fig. 2(a)). Similar results were obtained when the treatment temperature and pressure were varied. Cell nuclei, which have high polarity, seem not to dissolve in non-polar carbon dioxide. On the other hand, supercritical carbon dioxide that contained ethanol was able to extract cell nuclei. As shown in Fig. 2(c) and (d), cell nuclei in the tissue were completely removed from the extracellular matrix. Cell nuclei, which did not dissolve in either carbon dioxide or ethanol, appeared to be effectively solubilized in mixed fluid under the supercritical condition. More remarkable was the decellularization that was attained after a short period of treatment. Figure 2(d) demonstrates that cell nuclei were removed within 15 min when the supercritical carbon dioxide/ethanol system was used. Compared with an aqueous detergent system, the high diffusion coefficient and low viscosity of supercritical fluid will enable rapid access of the fluid into the extracellular matrix. As a result, rapid extraction of cell nuclei can be attained with this system. Because complete decellularization with other techniques is generally performed on a daily basis, supercritical fluid extraction for the same purpose can markedly shorten treatment time. These results show that cell nuclei can easily be removed in a short time without using chemicals such as surfactants and enzymes. A method for quantitative analysis of cell nuclei in supercritical carbon dioxide is not yet established. Future investigations of methods for evaluating the solubility of cell nuclei in supercritical carbon dioxide would be necessary to confirm the observations made in this study.

Figure 3 shows the variation in the percentage of residual phospholipids in the tissue after treatment with supercritical carbon dioxide/ethanol using a batch system. In this case, the operating pressure was fixed at 30 MPa. For comparison, data obtained from a similar system that did not contain ethanol are also shown. As

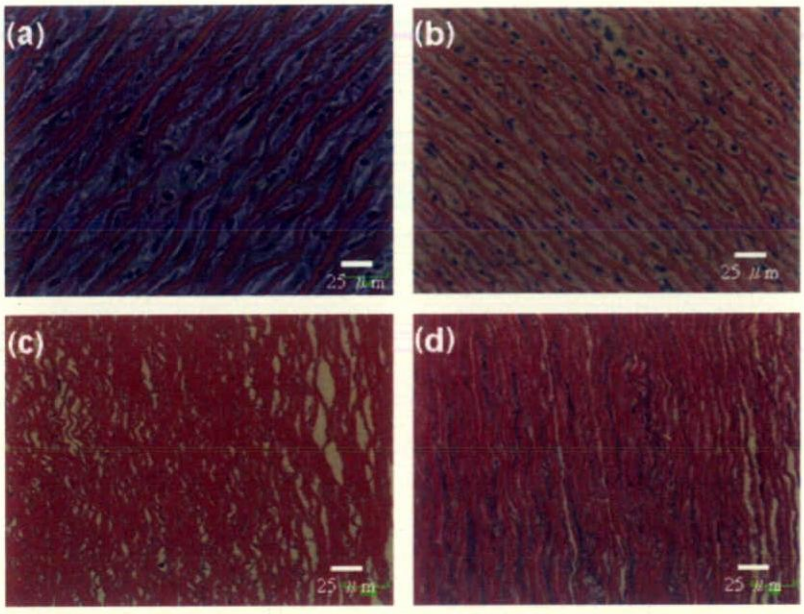

Figure 2. Hematoxylin-Eosin staining of porcine aorta treated in supercritical carbon dioxide. (Batch system): (a) native aorta; (b) aorta treated with supercritical carbon dioxide only; (c) aorta treated with supercritical carbon dioxide/ethanol for 1 h; (d) aorta treated with supercritical carbon dioxide/ethanol for 15 min.

described before, residual phospholipid from a donor may cause calcification after implantation. Therefore, complete removal of phospholipid is desirable during the decellularization process. As shown in Fig. 3, in the absence of ethanol, the system had little ability to extract phospholipid. The results showed that neither cell nuclei nor phospholipid appeared to dissolve in carbon dioxide alone, even when the system was in the supercritical condition. On the other hand, inclusion of ethanol in the system had an obvious effect on the extraction of phospholipid; data show that the percentage of residual phospholipid in the tissue decreased with increasing treatment time and reached a plateau. In this experimental condition, extraction of phospholipid could be attained by 30 min of treatment. Mixed supercritical fluid (carbon dioxide/ethanol), which rapidly reached the inside of the tissue, seems to have the potential to solubilize and extract both cell nuclei and phospholipid within a short time. However, complete extraction of phospholipid was not attained. Some phospholipid remained in the tissue even when the treatment was prolonged.

Figure 4 shows the effects of operating pressure on removal of phospholipid from the tissue. Operating time was fixed at 60 min. As shown in Fig. 4, the percentage of residual phospholipid in the tissue decreased with increasing pressure and reached a plateau. Because an increase in pressure results in an increase in solvent power, the solubility of high-polar phospholipid gradually increased with increasing pressure. The data indicate that the solvent power of a supercritical carbon dioxide/ethanol system at about 20 MPa was sufficient to solubilize phospholipid. Further increase in pressure has only an insignificant effect on the solubilization of phospholipid. As shown before, removal of the cell nuclei could also be attained at 20 MPa. From these data, it is concluded that 20 MPa is the threshold pressure for decellularization of tissue with a supercritical carbon dioxide/ethanol system. Unfortunately, residual phospholipid in the tissue did not reach zero. Some phospholipids remained even when the pressure was raised.

These results may be related to the operating system of the apparatus. The data shown in Figs 3 and 4 were obtained in experiments using a batch system. Because adsorption and desorption of solute in this system are

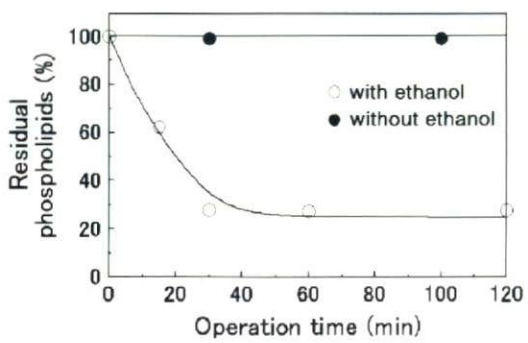

Figure 3. Effect of treatment time on the phospholipid removal from tissue in supercritical carbon dioxide (batch system).

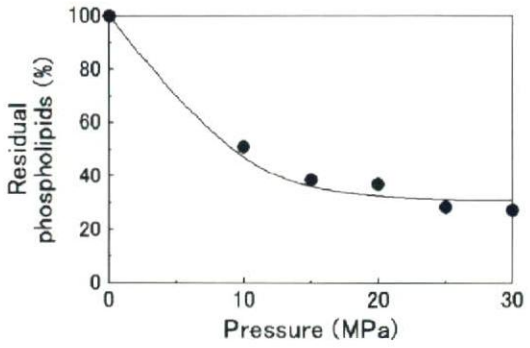

Figure 4. Effect of treatment pressure on the phospholipid removal from tissue in supercritical carbon dioxide (batch system).

in equilibrium with each other, complete removal of phospholipid in a single trial may be difficult. For the complete removal of phospholipid, repetition of the procedure is necessary. However, it is difficult to determine operating times for individual tissues, since tissues differ from each other. In addition, repetition of the operation is not favorable from the perspective of reducing operation time and retaining the mechanical strength of the tissue. In contrast, the use of a system that can control the continuous flow of the fluid may be effective in reducing the amount of residual phospholipids in the tissue. In the following experiments, data were collected from such a system.

Figure 5 shows variations in residual phospholipid in the tissue as a function of treatment time. In this case, the flow rate of the supercritical carbon dioxide/ethanol mixture was 1 mL min^{-1}. Overflowing fluid mixture was captured with a trap through a backpressure regulator. As shown in Fig. 5, phospholipid in the tissue was effectively removed by a short period of treatment. Comparison with the data in Fig. 3 shows that the time to reach a constant percentage of residual phospholipid with this system was shorter than the time required using the batch system. However, some phospholipids remained in the tissue. The amount of residual phospholipids was about 20% of the amount in native tissue and was only slightly different from that in the batch system. Similar data were obtained when the operating pressure was varied. From these data, fluid flow appears not to affect extraction efficiency, but improves extraction velocity.

In order to improve phospholipid removal, further investigation was undertaken taking into consideration the mass transfer rate of mixed fluid in the tissue. It is generally accepted that phospholipids within a tissue must migrate to the surface of the tissue if extraction is to succeed. In this step, the mass transfer rate of the fluid would be closely related to the migration of phospholipids. However, the mass transfer rate of mixed fluid within the tissue under the constant flow condition may be negligible compared with that on the outside. On the other hand, considerable mass transfer of mixed fluid within the tissue would be observed on increasing or decreasing pressure. In the process of increasing the pressure, mixed fluid

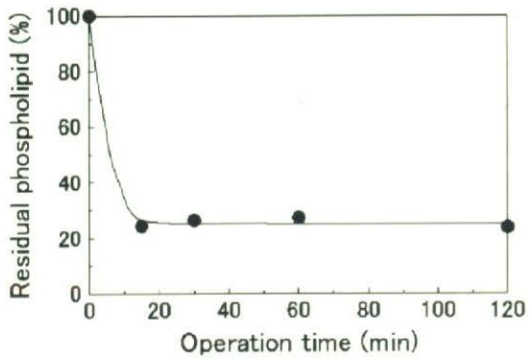

Figure 5. Effect of treatment pressure on the phospholipid removal from tissue in supercritical carbon dioxide (flow system).

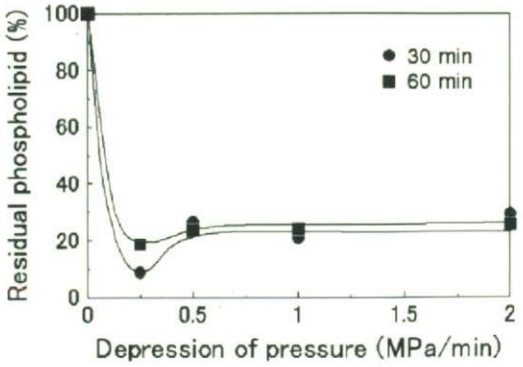

Figure 6. Effect of depression pressure on the phospholipid removal from tissue in supercritical carbon dioxide (flow system).

in the tissue will not move to the outside because the system is closed until the pressure reaches the desired value. In contrast, the process of decreasing the pressure favors the transfer of mixed fluid to the outside through the backpressure regulator. As a result, phospholipids solubilized in the mixed fluid also migrate to the outside of the tissue. In order to evaluate this hypothesis, carbon dioxide in the vessel was gradually released at a strictly controlled flow rate. In this case, pressure was decreased after treatment at constant pressure (30 MPa) for 30 or 60 min.

Figure 6 shows phospholipid removal as a function of pressure depression velocity. It is obvious that pressure depression velocity affects phospholipid removal. The migration velocity of phospholipids in the tissue was also determined at the time of depression of pressure. The data show that the most effective rate of reducing the pressure was 0.25 MPa min^{-1}. Phospholipid removal was lower at a pressure depression velocity of 0.25 MPa min^{-1} than at other rates shown in Fig. 6. A lower pressure depression velocity produces slower variation in the solubility parameter of the mixed fluid. This indicates that phospholipids have considerable opportunity to solubilize in a suitable polar fluid. From the data in Fig. 6, the depression velocity of 0.25 MPa min^{-1} seems to be the best value for both solubilization and migration of phospholipids. However, residual phospholipids did not reach zero throughout the range of depression velocities tested. As described before, continuous pressure depression provides variation in the solubility parameter of the mixed fluid. As a result, some of the phospholipids solubilized in the mixed fluid would be precipitated before migration to the outside of the tissue. Further detailed investigation to determine the best pressure depression velocity may lead to greater efficiency of phospholipid removal in this system.

Evaluation of mechanical properties of tissue

Figure 7 shows stress–strain curves for porcine aorta treated with the supercritical carbon dioxide/ethanol system. The operating pressure was fixed at 35 MPa. As shown in Fig. 7, mixed fluids seem to have no influence on the ultimate breaking strength of

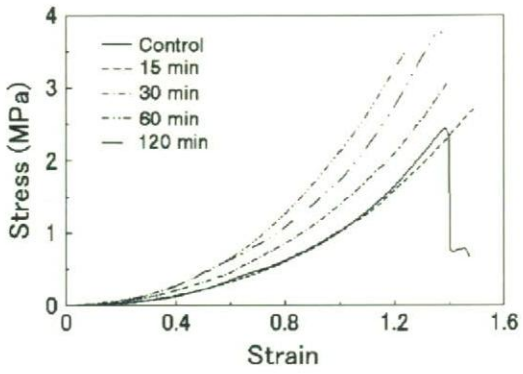

Figure 7. Stress–strain curves of porcine aorta treated in supercritical carbon dioxide (flow system).

the tissue. This suggests that collagen and elastin fibers in the extracellular matrix of the tissue are not broken even if the system is in a high-pressure condition. Because the investigations in this study were performed at a comparatively low temperature and pressure, carbon dioxide in the reaction vessel would not have had enough energy to break the chemical bond of protein fibers in the extracellular matrix. On the other hand, the slope of the curve in Fig. 7 increases with increasing operation time. Prolonging the operation time under the supercritical condition causes hardening of the tissue, which becomes considerable in the high-strain region. This result may be related to tissue dehydration. Because fresh ethanol/carbon dioxide mixture constantly flowed in the reaction vessel for appropriate times, the water content in the tissue decreased with time. As a result, the stereo structure of the protein fibers in the extracellular matrix may have gradually degenerated.

Figure 8 shows the effects of operating pressure on the mechanical strength of porcine aorta. The operation time was fixed at 60 min. The data are similar to those in Fig. 7. The ultimate breaking strength of the tissue was not decreased. On the other hand, an increase in operating pressure causes hardening of the tissue. This result may also be explained in terms of variation in the stereo structure of the protein fibers. An increase in pressure under constant temperature results in an increase in the

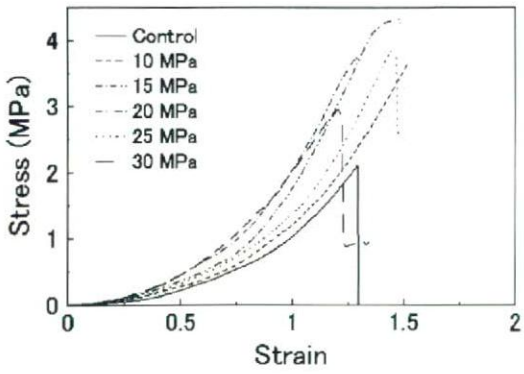

Figure 8. Stress–strain curves of porcine aorta treated in supercritical carbon dioxide (flow system).

density of carbon dioxide. The ethanol content is higher at higher densities of carbon dioxide. As a result, water in the tissue dehydrates easily under the condition of higher carbon dioxide density.

These findings are supported by the data in Fig. 9, which shows the weight variation of the aorta after treatment with supercritical carbon dioxide/ethanol. As shown in the curves identified by circles, the weight of porcine aorta decreased to about 70% that of the native tissue after treatment with only supercritical carbon dioxide/ethanol. The water in the tissue was obviously removed and dissolved in the fluid mixture. However, the weight of the aorta did not recover compared with that of the native tissue even if the aorta was soaked in aqueous solution. Weight recovery was about 90% that of the native weight. This result suggests that treatment with supercritical carbon dioxide/ethanol reduces the possibility of incorporation of water in the inner part of the tissue. Insignificant variation in the stereo structure of the tissue due to dehydration with ethanol may cause slight contraction of the tissue.

These results show that the ethanol in supercritical carbon dioxide causes tissue dehydration. Hardening of the tissue due to dehydration became considerable in the high-strain region. Fortunately, the strain that an organ is subjected to during practice is represented toward the lower end of the abscissa (Figs 7 and 8). In this strain region, differences in hardening due to differences in operating conditions will be negligible. In addition, the ultimate breaking strength of the tissue was equivalent to that of the native standard. The most important role of decellularized tissue is to retain mechanical strength as a scaffold. Tissue prepared in this study had satisfactory characteristics in this respect. In order to investigate the strength of tissue in detail, *in vivo* evaluation in a long-standing implantation is necessary.

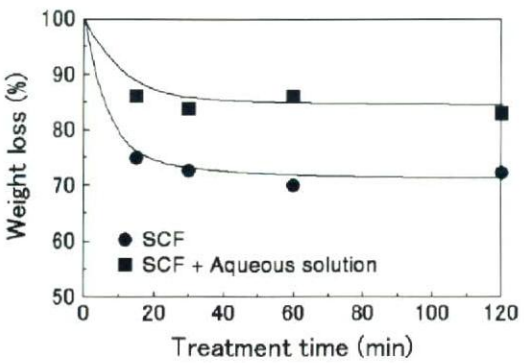

Figure 9. Comparison of weight recovery of porcine aorta before and after the treatment in supercritical carbon dioxide.

CONCLUSIONS

Supercritical carbon dioxide that contained a small amount of ethanol was used as an extraction medium to remove the cells from artificial tissue. The cell nucleus and cell membrane in the tissue could be effectively removed within 20 min under mild conditions (15 MPa, 37 °C). No decrease was observed in the mechanical strength of the tissue because of treatment under the supercritical condition.

The safety level of the recipient is high if the decellularized tissue is processed using supercritical carbon dioxide, because undesirable chemicals such as surfactants and aldehydes are not necessary. In addition, decellularized tissue can be obtained in an absolutely dry condition. This means that the decellularized tissue can be preserved semipermanently until use. Further evaluation for pathogens, such as endogenous viruses and prions, would increase the potential of the supercritical fluid extraction method for preparing acellular artificial tissue.

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