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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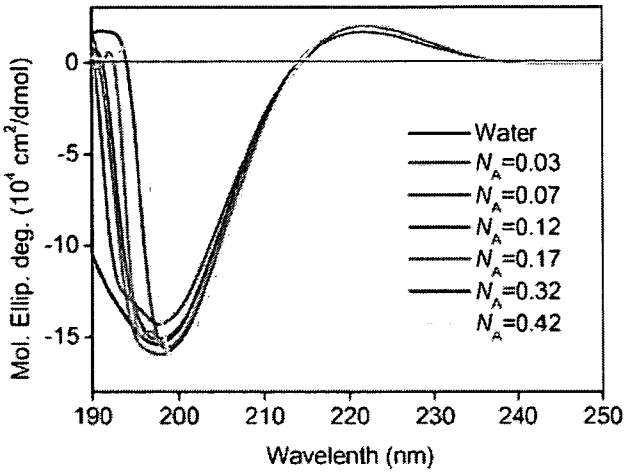

Figure 1. CD spectra of the collagen microfibrils under various ethanol mole concentrations.

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,

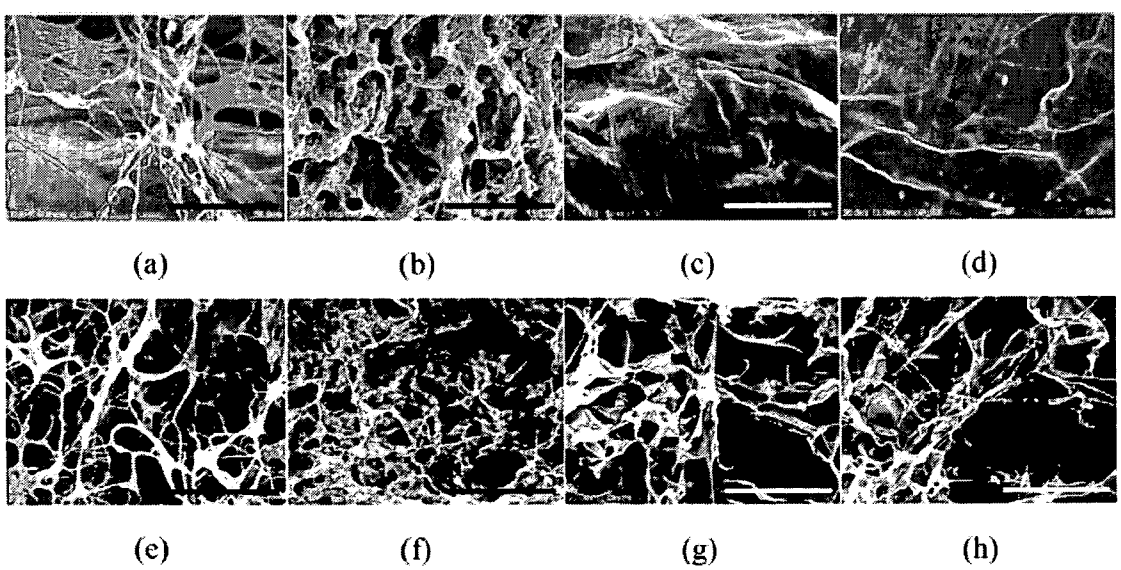

Figure 2. Morphology of collagens after immersing in ethanol/water mixed solvents of different concentrations. (a) Water, (b) $N_A \approx 0.07$, (c) $N_A \approx 0.17$, (d) $N_A \approx 0.32$, (e) $N_A \approx 0.42$, (f) $N_A \approx 0.55$, (g) $N_A \approx 0.73$, and (h) ethanol. Single bar indicates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

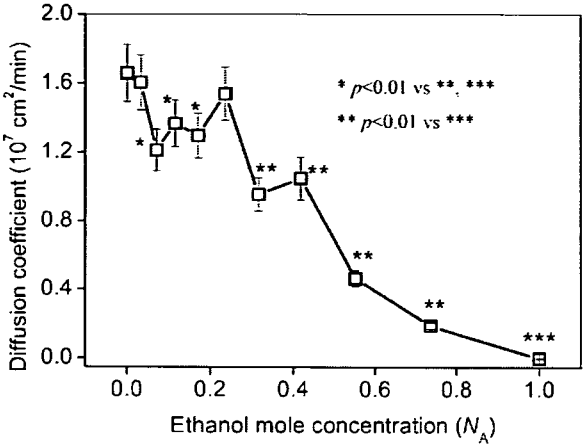

Figure 3. Change in the diffusion coefficient of ethanol in collagen gel according to ethanol mole concentrations.

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

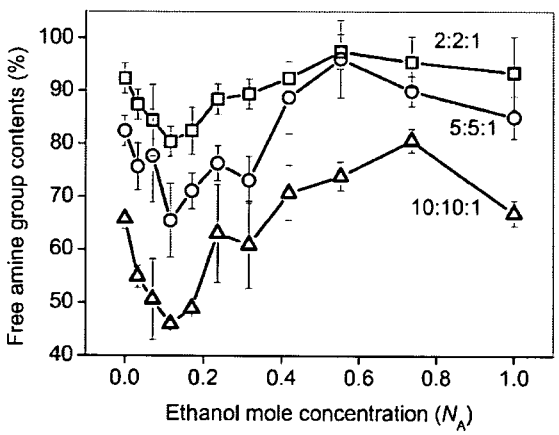

Figure 4. Change in the free amine group contents of collagen gel according to ethanol mole concentrations.

$N_A \approx 0.24$ (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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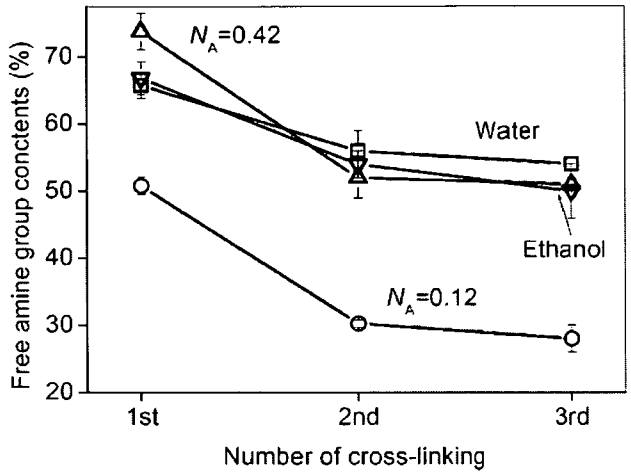

Figure 5. Change in the free amine group content of collagen gel by the re-crosslinking procedure in different solvents.

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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Preparation and characterization of the condensed plasmid DNA by high hydrostatic pressurization

Tsuyoshi Kimura[1], Kana Horiuchi[2], Kimio Kurita[2], Kwangwoo Nam[1], Tsutomu Ono[3], Hidekazu Yoshizawa[3], Tsutomu Furuzono[4], Toshiya Fujisato[5], Akio Kishida[1]

[1]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan, [2]Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, 1-8-14, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8308, Japan, [3]Gradual School of Environmental Science, Okayama University, 3-1-1, Tsushimanaka, Okayama 700-8530, Japan, [4]Department of Biomedical Engineering, National Cardiovascular Center Research Institute,5-7-1, Fujishirodai, Suita, Osaka 656-8565, Japan, [5]Division of Biomedical Engineering, Osaka Institute of Technology, 5-16-1 Omiya, Asahi-ku, Osaka , Japan

It was found that the condensation of super-coiled plasmid DNA was induced by high hydrostatic pressurization. The spherical structure of the condensed plasmid DNA was observed for atomic force microscopic observation. The degree of condensation was increased with increasing the pressurizing strength and time. The pressure-condensed plasmid DNA was effectively recognized by transcriptional factors.

For non-viral gene delivery system using plasmid DNA, plasmid DNA has been directly injected into local regions *in vivo* [1] or condensed by cationic compounds, such as cationic polymers, peptides, and lipids, which can interacted with DNA electrostatically, and then delivered into cells *in vitro* and *in vivo*.[2] Although the former method is simple and safe, the level of transgene expression is relatively low and restricted to the injection site due to its low stability. The latter methods show the relative high transfection efficiency *in vitro* due to the high stability of condensates, whereas the cytotoxicity of cationic materials is one of essential problems *in vitro* and *in vivo*. Therefore, for safer, more stable and efficient delivery system of plasmid DNA, it is necessary to condense plasmid DNA with a less cationic compound or without one.

It is well known that pressure, which is one of intensive variables in thermodynamic as well as temperature and concentration, strongly affects on protein structure formed via various weak interactions, such as electrostatic, hydrophobic and hydrogen bonding interactions,[3] and that the hydrogen bond is strengthen than the other interaction under high pressure condition.[4] From this fact, we previously reported that the nanoparticles and hydrogels of polyvinyl alcohol (PVA) were formed via hydrogen bonds by high hydrostatic pressurization.[5] Also, PVA nanoparticles and hydrogels interacting with DNA via hydrogen bonds could be delivered into cells[6] and release DNA[7], respectively. In relation to the effect of pressure on nucleic acid, which is one of hydrogen bonded bio-polymers, it was reported that the stability of a DNA duplex was increased with increasing pressure.[8] Also, super-coiling of a relaxed plasmid DNA was induced by the elevated pressure because of pressure-increasing of the winding and twisting of DNA helix.[9] In the present study, we hypothesized that the super-coiled plasmid DNA could be condensed by high hydrostatic pressurization, and investigated the change in the tertiary structure of plasmid DNA having the super-coiled and relaxed forms through various physicochemical methods. Also, the recognition of the pressurized plasmid DNA by transcription factor was examined in order to be applicable for gene delivery.

Plasmid DNA encoding luciferase gene under T7 promoter (pT7-luc, Promega Co., Ltd.) was used. The aqueous solution of pT7-luc was prepared at the concentration of 20 ng/μl and hydrostatically pressurized at different atmosphere ranging from 2,000 to 10,000 atm, and at 40 °C for various times from 1 to 20 min using high pressurizing machine (Dr.chef, Kobe Steel Co., Ltd.). After pressure removal, the obtained solutions were analyzed by dynamic light scattering (DLS) measurement (Nano-Zs, Malvern Instruments Ltd.). Their size distributions by intensity were overlaid in Figure 1. Table 1 shows the hydrodynamic size of the pressurized pT7-luc at different atmosphere for 5 min. For the non-pressurized pT7-luc, the super-coiled and relaxed forms of pT7-luc were detected at approximately 94±2 and 624±60 nm, respectively. The hydrodynamic size of them was decreased with increasing pressure, and then measured at approximately 25±2 and 108±9 nm, respectively, for the pressurization at 10,000 atm. This change in the latter size from 624 to 108 nm was regarded as super-coiling of the relaxed pT7-luc by the hydrostatic pressurization.[9] When the relaxed pT7-luc, which was enzymatically prepared using topoisomerase I, was treated by the pressurization at 10,000 atm for 10 min, the super-coiled pT7-luc was measured at approximately 138±19 nm by DLS measurement (data not shown). Thus, the decrease in the size of the super-coiled pT7-luc from 94 to 25 nm suggests that the super-coiled pT7-luc was condensed by high hydrostatic pressurization. At constant pressure at 10,000 atm, a long period of pressure treatment effectively induced the condensation of super-coiled pT7-luc and super-coiling of a relaxed pT7-luc (data not shown). From these results, it

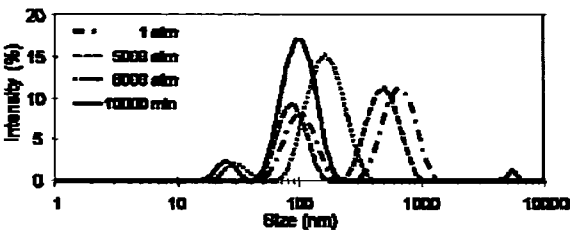

Figure 1. DLS measurement of the pressurized pT7-luc at various atmospheres and 40 °C for 5 min.

Table 1. Hydrodynamic size of the pressurized pT7-luc at various atmospheres and 40 °C for 5 min.

Pressure (atm)	Hydrodynamic size (nm)	
	Peak 1	Peak 2
1	94 ± 2	624 ± 60
5000	93 ± 6	529 ± 25
8000	33 ± 8	168 ± 21
10000	25 ± 2	108 ± 9

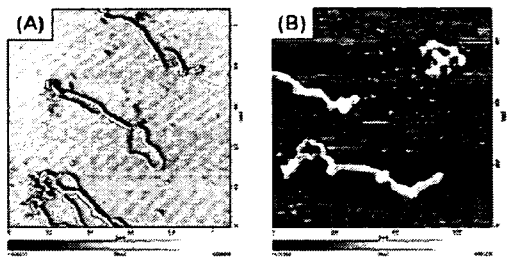

Figure 2. AFM images of pT7-luc (A) without the pressurization and (B) with the pressurization at 10,000 atm for 5min.

Table 2. Fluorescent intensity and DLS measurements of the pT7-luc intercalated with EtBr pressurized at various atmospheres for 5min.

Pressure (atm)	Fluorescent [a] intensity	Hydrodynamic size (nm) [b] Peak 1	Peak 2
1	15.3 ± 0.2	133 ± 16	924 ± 147
3000	15.3 ± 0.8	118 ± 16	515 ± 65
5000	13.5 ± 1.9	100 ± 23	566 ± 205
8000	12.7 ± 0.5	119 ± 7	318 ± 125
10000	13.0 ± 2.4	82 ± 58	280 ± 126

[a] Ex: 511nm, Em: 603nm, [b] DLS measurement

suggests that the tertiary structure of plasmid DNA, condensation and super-coiling, could be controlled using pressurizing process and be remained after pressure removal.

Atomic force microscope image of the pT7-luc without the pressurization on mica in air is shown in Figure 2 (A).[10] The fibrous super-coil form of pT7-luc that was highly twisting was mainly observed. The super-coil form being partially opened was also exhibited. On the other hand, for the pressurized pT7-luc at 10,000 atm for 5 min, the super-coil form having partially globule was observed (Figure 2 (B)). Further, the entirely spherical structure of the pressurized pT7-luc was also observed. It is assumed that the pressure-induced twisting and winding of plasmid DNA caused the condensation of super-coiled plasmid DNA.

Ethidium bromide (EtBr), which can intercalate into DNA double helix, resulting in a significant increase in the EtBr fluorescence intensity, is commonly used to monitor the DNA condensation induced by a complex formation with cationic polymers, in which the EtBr fluorescent intensity reduced due to being excluded.[11] The plasmid DNA intercalated with EtBr at an equimolar ratio per base pair was pressurized at various atmospheres and subjected by fluorescent intensity measurement (FP-6500, JASCO Co., Ltd., Ex: 511nm, Em: 603nm) after pressure removal (Table 2). Although the EtBr fluorescent intensity was decreased with increasing pressure, even for the pressurization at 10,000 atm, the EtBr fluorescent intensity reduced to only about 85 % of that of the non-pressurized pT7-luc. The hydrodynamic size of the pressurized pT7-luc with EtBr was ineffectively decreased compared to that of the pressurized pT7-luc without one. These results suggest that the EtBr intercalating into plasmid DNA inhibited the pressure-induced condensation and super-coiling of plasmid DNA. Also, it is considered that

Figure 3 shows the activity of luciferase expressed from the pT7-luc treated with the pressurization at various atmospheres for 5min in cell-free translation and translation

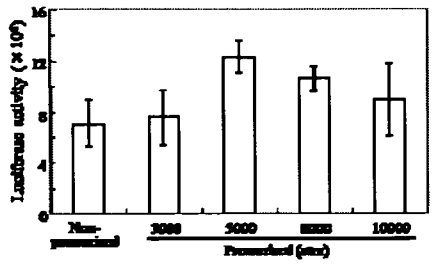

Figure 3. Transcription and translation activity of the pressurized pT7-luc at various atmospheres for 5min in cell-free system.

system (TNT Coupled Reticulocyte Lysate System, Promega Co., Ltd.).[12] The luciferase activity of the pressurized pT7-luc at 5,000 atm was increased about 1.8 times compared to the non-pressurized one. Although more increasing of pressure decreased the luciferase activity, indicating that the pressurized plasmid DNA was applicable for gene delivery.

Acknowledgement

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10. The 10 µl of pT7-luc solutions at the concentration of 20 µg/ml with or without the pressurization at 10,000 atm for 5 min was injected onto mica for 5 min, thoroughly rinsed with deionized water, twice, and then blown dry with compressed air. AFM studies were preformed using the SPA-300 scanning probe microscope (Seiko Instruments Inc.) operated in tapping mode.
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12. Two µl of the pT7-luc solutions (20 ng/µl) with the high pressurization from 2,000 to 10,000 atm for 5 min was mixed with eight µl of rabbit reticulocyte lysate and incubated at 30 °C for 1 h. The five µl aliquots were added to a luciferase reaction buffer (Luciferase Assay System, Promega Co., Ltd.), and the light emission was measured on a luminometer (AB-2200, ATTO Co., Ltd.,)

P-13

高圧凝縮ＤＮＡの基礎的検討と遺伝子デリバリーへの応用

○木村剛[1]、吉澤秀和[2]、古薗勉[3]、藤里俊哉[4]、岸田晶夫[1]
[1]東京医科歯科大学・生体材料工学研究所、[2]岡山大学・環境理工学部、[3]国立循環器病センター研究所・生体工学部、[4]国立循環器病センター研究所・再生医療部

【緒言】

DNA、RNAなどの核酸は、遺伝子情報を担う塩基配列(一次構造)とそれらの遺伝情報の保持・発現のための二次構造が重要である。核酸の高次構造は、温度、圧力、pH、塩濃度などの外部環境に影響されることが知られている。これまで、核酸への圧力の影響として、低分子量DNAの構造変化や、RNA(リボザイム)活性の低下が知られている。本研究では、遺伝子導入における遺伝子発現を調節することを目的として、圧力印加によるDNA構造変化およびその機能について検討した。

【方法】

DNAとしては、1kbラダーDNAとプラスミドDNA（pT7-Luc）を用いた。超高圧印加装置(Dr.chef(神戸製鋼所))を用いて、温度40℃、圧力3,000、5,000、8,000、10,000気圧、印加時間1、5、20分間と異なる条件にて高圧処理を行った。常圧に戻した後、DNA溶液をTm測定、CD測定、DLS測定、蛍光強度測定にて解析した。また、高圧処理によるDNAの機能解析として、ウサギ網状赤血球を用いた無細胞系転写・翻訳により検討した。

【結果・考察】

超高圧印加によるラダーDNAの構造変化は、CD測定、Tm測定では示されなかった。一方、プラスミドDNAでは、CD測定の結果から構造変化が示唆され、また、DLS測定よりプラスミドDNAのサイズ減少が示された。プラスミドDNAサイズへの圧力の影響として超高圧処理時間の影響を詳細に検討した結果、圧力の増加と圧力印加時間の延長に伴い、DNAサイズが減少することが示された。次に、DNAインターカレーターであるエチジウムブロマイド(EtBr)を用いて、プラスミドDNA構造への圧力影響について検討した。圧力増加に伴う蛍光強度の減少が示された。これは、DNAにインターカレートしていたEtBrが外れたことによる蛍光強度の減少と考えられ、高圧印加によるDNAの構造変化を示す。また、インターカレートしたプラスミドDNAにおいてもサイズの減少が示された。以上より、プラスミドDNAは高圧印加により凝縮すると考えられる。部分的あるいは全体的に凝縮され、オープンサークルからスーパーコイルへの転移、さらにはよりきつく巻かれたスーパーコイルになると考えられる。これを判断するために、原子間力顕微鏡(AFM)による視覚的な観察を検討している。また、凝縮したDNAの機能について調べるため無細胞系転写・翻訳を行った結果、圧力による転写・翻訳活性の向上が示された。一方、処理時間による活性への影響は示されなかった。活性の促進は、高圧処理により転写因子に認識されやすい構造に変化したことによると考えられる。本研究は、厚生労働科学研究費の補助を受けて行われた。

3Pd122

高圧印加による PEG/多糖水性二相形成の促進

東医歯大生材研　○木村剛　　日大理工　三浦義之・栗田公夫
岡山大環境理工　六雄伸悟・吉澤秀和　　国循セ研　藤里俊哉
東医歯大生材研　岸田晶夫

<緒言>

２種類の水溶性高分子混合溶液における相分離形成（水性二相）が知られている。水性二相形成は、用いる高分子の分子量、濃度に依存し、また、塩濃度、温度、pH などの外部環境の影響を強く受ける。本研究では、温度と同様の示強パラメータである圧力に着目し、圧力印加による水性二相形成に関して検討した。ここでは、代表的な水性二相系である PEG-Dextran 混合溶液にて検討した。

<実験>

ポリエチレングリコール（PEG ; Mw : 6000、8000）、デキストラン（Dex ; Mw : 32,000～45,000、60,000～90,000、100,000～200,000、500,000）を用いた。各 10%(w/v)水溶液を調製し、1:1 の割合で混合した後、25℃で 10,000 気圧、10 分間超高圧印加処理した。処理液を、目視による観察、動的光散乱(DLS)測定、示差走査熱量(DSC)測定、NMR 測定、旋光度測定にて構造体の物性解析を行った。

<結果・考察>

PEG 水溶液、Dex 水溶液への超高圧印加処理では、目視による変化は確認されず、透明溶液であった。DLS 測定においてもサイズの変化は示されなかった。一方、PEG/Dex 混合溶液においては、PEG の分子量に依らず、低分子量の Dex32,000～45,000 では透明溶液であり、Dex60,000～90,000 および 100,000～200,000 では青白色を呈した。エマルジョンが形成されたことによる散乱と考えられる。また、Dex500,000 は PEG との混合により白濁溶液となり、その後の放置により二相分離した。PEG/Dex 混合液への超高圧印加処理では、Dex60,000～90,000 および 100,000～200,000 にて二相分離が形成され、下相にて青白色を呈した。Dex500,000 では、白濁溶液状態および二相形成状態への超高圧印加処理のいずれの場合も二相分離を形成し、上下相とも透明溶液であった。従って、この現象は超高圧による PEG と DEX から成る構造体の形成に伴う見かけの分子量の増加による水性二相形成と考えられる。生じた二相分離の下相部の溶液の DLS 測定（25℃）では、いずれの場合も粒子径の増加が示された。また、加熱処理による粒子径の減少が DLS 測定にて示され、PEG/DEX のエマルションへの超高圧印加処理により、水素結合を介した新規 PEG/Dex 複合体が形成されたと考えられる。これらを詳細に検討するため、上下相溶液の NMR 測定を行った。上相では PEG に帰属するピークが、下相では PEG と Dex に帰属するピークが検出され、上相には PEG のみが、下相には PEG と Dex が存在することを示された。また、水素結合阻害剤である尿素添加系においては、超高圧処理に誘起される二相分離が形成されなかったことから、水素結合を介した PEG/Dex 複合体の形成が強く示唆された。以上の結果より、高圧印加による PEG/多糖水性二相形成の促進が示された。本研究は、厚生労働科学研究費および文部科学研究費の補助を受けて行われた。

Enhancement of aqueous two phase separation of PEG-polycarbonate by high pressurization

Tsuyoshi KIMURA[1], Yoshiyuki MIURA[2], Kimio KURITA[2], Shingo MUTSUO[3], Hidekazu YOSHIZAWA[3], Toshiya FUJISAO[4], Akio KISHIDA[1]([1]Tokyo Medical and Dental Univ., 2-3-10 Kanda-surugadai, Chiyoda-ku, Tokyo 101-0062, [2]Nihon Univ., [3]Okayama Univ., [4]National Cardiovascular Center Reserch Institute)

[1]TEL&FAX: +81-3-5280-8029, E-mail: kimurat.fm@tmd.ac.jp

Key Word: high pressurization / aqueous phase separation / PEG / Dextran / DLS

Abstract: In this study, the aqueous phase system of PEG and dextran was studied under high pressure condition. The mixed solutions of PEG and dextran having various molecular weights were treated with ultra high pressurization (10,000 atm, 25 degree, 10min). Various analyses, such as DLS, NMR and DSC measurements, were carried out. We found that the assembling of poly(ethyleneglycol) (PEG) and dextran was induced by ultra high pressurization. The assembly of PEG and dextran induced the formation of aqueous two-phase system. There was the assembly of PEG and dextran and PEG molecules in the lower and upper phase, respectively.

2Pf134

超高圧誘起 PVA ハイドロゲルの調製と物性に関する研究

日大院理工　○三浦義之・栗田公夫、東医歯大生材研　木村剛、
岡山大環境理工　六雄伸吾・吉澤秀和、国立循環器セ　藤里俊哉、
物材機構　小林尚俊、東医歯大生材研　岸田晶夫

<緒言>

超高圧条件下では、疎水性相互作用が弱まり、水素結合が強調されることが報告されている。我々はこの点に着目し、水酸基を有する水素結合性分子への圧力印加による水素結合を介する構造体の創出について検討している。これまで、ポリビニルアルコール(PVA)、ポリエチレングリコール(PEG)、アガロース、デキストランを用いて、それらの種々の条件下での静水圧処理を施した結果、PVA にてハイドロゲルが得られた。本研究では、PVA ハイドロゲルの作製および物性について詳細に報告する。

<実験>

PVA(重合度:1700,鹸化度:99.8%)の水溶液およびジメチルスルホキシド(DMSO)/水(DMSO:水=80:20)混合液を様々な濃度で調製した。種種の温度(25~40℃)・圧力(1,000~10,000 気圧)・時間(0~30 分)にて高圧処理装置(Dr.CHEF;(株)神戸製鋼所)を用いて、高圧処理を行った。また、PVA ハイドロゲルの一般的な作成方法である凍結融解法を用いて PVA ハイドロゲルを作製した。得られた PVA ハイドロゲルのマクロ・ミクロ観察、透過度、膨潤度、力学強度測定にて物性解析を行なった。

<結果・考察>

従来から PVA 溶液（DMSO 混合系）を凍結・融解することにより PVA ゲルが得られる。－２０℃にて２時間インキュベートし、室温にて３０分間放置し、これを１サイクルとして１０サイクルを繰り返して凍結融解 PVA ゲルを得た。得られた凍結融解ゲルは、透明性が高く、機械的強度にも優れていた。一方、PVA 水溶液への超高圧印加処理にて得られた PVA ゲルは白色ゲルであり、DMSO 混合液では透明ゲルであった。溶媒により水素結合結晶領域のサイズ、密度が異なっていたためと考えられる。圧縮試験の結果、表面強度は凍結融解法で調製したゲルの方が強く、ゲル内部の強度は高圧で調製したゲルの方が強いことが示された。さらに高圧処理によるゲルは、PVA 濃度、処理温度および処理圧力に依存的にゲルの物性が異なった。凍結融解法ではコントロールが困難である様々な特性を高圧処理により制御の可能性が示唆された。発表では、種々の条件による PVA ゲル形成についてさらに検討し、また、力学強度、透過性等の物性についても詳細に検討したので報告する。

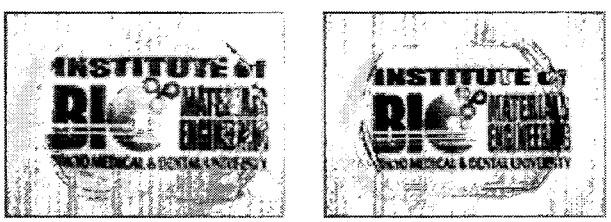

Fig1. Photos of PVA hydrogels using ultra-high pressurization or repeated freeze/thaw method. (Left)UHP method. PVA solution was treated under the UHP condition(10,000atm, 10degrees) for ten min. (Right)Repeated freeze/thaw method.

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Preparation and characteristics of PVA hydrogels induced by ultra-high pressurization

Yoshiyuki MIURA[1], Kimio KURITA[1], Tsuyoshi KIMURA[2], Shingo MUTSUO[3], Hidekazu YOSHIZAWA[3], Toshiya FUJISAO[4], Hisatoshi KOBATASHI[5], Akio KISHIDA[2]([1]Nihon Univ., 1-8-14 Kanda –surugadai, Chiyoda-ku, Tokyo 101-0062, Japan,[2]Tokyo Medical and Dental Univ., [3]Okayama Univ., [4]National Cardiovascular Center Reserch Institute, [5]National Institute of Materials Science)

[2]TEL&FAX: +81-3-5280-8029, E-mail: kimurat.fm@tmd.ac.jp

Key Word: ultra high pressure / hydrogel /

Abstract: In this study, the preparation and characteristics of PVA hydrogel induced by ultra-high pressurization was investigated. PVA hydrogel which has transparent was obtained by high hydrostatic pressurization at more than 8,000atm for 10min. The formation of the PVA hydrogels was depended on the pressuring temperature and the higher strength of pressurization induced the gelation of PVA. Also, PVA with higher concentration tended to form the hydrogel. It was clear that the obtained hydrogels was mediated by hydrogen bonding interaction.

1Pa177

高圧凝縮ＤＮＡの調製と機能解析

東医歯大生材研　○木村剛　日大理工　堀内可奈・三浦義之・栗田公夫
岡山大環境理工　吉澤秀和　国循セ研　藤里俊哉　　東医歯大生材研　岸田晶夫

<緒言>

無機・有機化学，医療，食品分野などでの高圧技術の利用が進んでおり，タンパク質，多糖への圧力効果を中心とする高圧バイオサイエンスが基礎研究として進められている．タンパク質，多糖，核酸などの生体高分子の構造に関する研究は，生体反応のメカニズムを解明し，生体機能を制御する上で重要である．DNA，RNA などの核酸は，遺伝子情報を担う塩基配列(一次構造)とそれらの遺伝情報の保持・発現のための二次構造が重要である．核酸の高次構造は，温度，圧力，pH，塩濃度などの環境条件に影響されることが知られている．これまで，核酸への圧力の影響として，DNA のコンホメーション変化や，圧力依存的に RNA(リボザイム)の活性が低下することがわかっている．しかし，DNA への高圧処理における活性については検討されていない．そこで本研究では，遺伝子導入における遺伝子発現を調節することを目的に，圧力印加による DNA 二次構造変化およびその機能について検討した．

<実験>

試料には 1kb ラダーDNA とプラスミド DNA(pT7-Luc)を用いた．超高圧印加装置を用いて，温度 40℃，圧力 3,000，5,000，8,000，10,000 気圧，印加時間 1，5，20 分間と異なる条件にて高圧処理を行った．常圧に戻したときの DNA 溶液を Tm 測定，CD 測定，DLS 測定，蛍光強度測定にて解析した．また，高圧処理による DNA の機能解析として，ウサギ網状赤血球を用いた無細胞系転写・翻訳により検討した．

<結果・考察>

CD 測定，Tm 測定の結果から，超高圧印加によるラダーDNA の構造変化は示されなかった．一方，プラスミド DNA では，CD 測定の結果から構造変化が示唆された．これは，環状であるためラダーDNA よりコンホメーションの変化性が大きいことによると考えられる．DLS 測定により慣性半径を調べたところ，超高圧印加による DNA サイズの減少が示された．さらに，プラスミド DNA サイズへの圧力の影響と超高圧処理時間の影響を調べた結果，圧力の増加と圧力印加時間の延長に伴い，DNA サイズが減少することが示された．次に，超高圧による影響でプラスミド DNA の構造変化が示唆されたことから，DNA インターカレーターであるエチジウムブロマイド(EtBr)を用いてプラスミド DNA への圧力の影響を調べた．圧力増加に伴う蛍光強度の減少が示された．これは，DNA にインターカレートしていた EtBr が外れたことによる蛍光強度の減少と考えられ，高圧印加による DNA の構造変化を示す．また，インターカレートしたプラスミド DNA においてもサイズの減少が示された．以上より，プラスミド DNA は高圧印加により凝縮すると考えられる．部分的あるいは全体的に凝縮され，オープンサークルからスーパーコイルへの転移，さらにはよりきつく巻かれたスーパーコイルになると考えられる．これを判断するために，原子間力顕微鏡(AFM)による視覚的な観察を検討している．凝縮した DNA の機能について調べるため無細胞系転写・翻訳を行った結果，圧力による転写・翻訳活性の向上が示された．一方，処理時間による活性への影響は示されなかった．活性の促進は，高圧処理により転写因子に認識されやすい構造に変化したことによると考えられる．本研究は、厚生労働科学研究費の補助を受けて行われた。

Preparation and characteristics of DNA molecules condensed by high pressurization

Tsuyoshi KIMURA[1], Kana HORIUCHI[2], Yoshiyuki MIURA[2], Kimio KURITA[2], Hidekazu YOSHIZAWA[3], Toshiya FUJISAO[4], Akio KISHIDA[1] ([1]Tokyo Medical and Dental Univ., 2-3-10 Kanda–surugadai, Chiyoda-ku, Tokyo 101-0062, [2]Nihon Univ., [3]Okayama Univ.,[4]National Cardiovascular Center Reserch Institute)
[1]TEL&FAX: +81-3-5280-8029, E-mail: kimurat.fm@tmd.ac.jp

Key Word: high pressurization / DNA/transcription and translation/

Abstract: We studied structure and functions of nucleic acids treated with high pressurization. DNA (1kb ladder molecular weight maker) was pressurized at 3000, 5000, 8000, 10,000 atms and 40 degree for 1, 5, 20 min. CD and Tm analyses revealed that there was no change for the structure of ladder DNA. On the other hand, when plasmid DNA (pT7-luc) was pressurized under conditions described above, the size of plasmid DNA was decreased with increasing the strength and period of pressurization. The inhibition test of intercalating of EtBr was carried out. The intensity of EtBr was decreased with increasing the strength of pressurization, indicating that the change of stacking of plasmid DNA.

Assembling of hydrogen-bonding-polymers using high pressure technology

Tsuyoshi Kimura[1], Kwangwoo Nam[1], Tsutomu Ono[2], Hidekazu Yoshizawa[2], Toshiya Fujisato[3], and Akio Kishida[1].
[1]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental Unisersity
Chiyoda-ku, Tokyo 101-0062, Japan
Tel:+81-3-5280-8029 Fax:+81-3-5280-8028
E-mail:kimurat.fm@tmd.ac.jp
[2] Department of Material and Energy Science, Okayama University,
[3] Department of Biomedical Engineering, Osaka Institute of Technology

Introduction

Pressure technology is applied for many fields, such as inorganic, organic and food processing. Also, in life science, high pressure effects on the denaturation of proteins[1] and other bio-compounds[2] and thermo-sensitive synthetic polymers[3,4], which are used as a model of bio-molecules, have been investigated because pressure is one of intensive variables in thermodynamic as well as the concentration and temperature. It was known from high pressure-induced protein denaturing studies that hydrogen bond was strengthen under high pressure condition rather than hydrophobic and electrostatic interactions[5,6]. From these facts, we hypothesized that assembly of hydrogen-bonding-polymers should be formed via hydrogen bond using high pressure technology. In this study, poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG) and dextran (Dex) were used as models of hydrogen-bonding polymers and were pressurized at various conditions, pressure strength, pressuring time, temperature, in order to investigate whether polymeric assemblies are formed by high pressurization. In other word, the aim of this study is the development of assembly method of hydrogen-bonding-polymers using ultra-high pressure technology.

Results and Discussions

PVA (Mw:74800, Kuraray), PEG(Mw: 6000 and 8000, Wako), and dextran (Mw: 32000-45000, 60000-90000, 500000, Wako) were used as hydrogen-bonding-polymers. The aqueous solutions of them were prepared at different concentrations and were hydrostatically pressurized at various conditions of pressure strength and pressuring time. Although PVA solution of 0.01w/v% was still translucent through the pressurization at 10,000 atm for 10min, the formation of nano-particles having the average diameter of 200 nm was confirmed by SEM observation. The morphology of PVA assembly depended on the PVA concentration and micro-particles, gels were formed with increasing concentration through pressurization described above. These results suggest that the pressure induced PVA assembly via the intra, inter- hydrogen bonds between PVA molecules. In order to investigate the pressure effect on the assembling of PVA, the aqueous PVA solution of 5 w/v% was pressurized at various levels of pressure. The size of particles was increased with increasing of pressure when the pressurized PVA solutions were measured by DLS measurement. Also, it was found that the assembling of PVA was promoted by prolongation of the pressurizing time. From these results, it was suggested that the assembly of PVA was controlled by high pressurization.

When aqueous solutions of PEGs or Dexs at 10 w/v% concentration were hydrostatically pressurized at 10,000 atm for 10 min, they were still clear solution, of which there was no change for DLS measurement. When PEG (Mw: 6000) solution were mixed with Dex (Mw: 32000-45000 and 60000-90000) solution at volume ratio of 1 to 1 (final concentration of 5 w/v%, respectively), the solutions with light scattering were obtained, suggesting the formation of micro emulsions of them. After pressurization at 10,000 for 10 min, aqueous two-phase separation having light scattering in lower phase was obtained for both cases (Fig 1), whereas no aqueous phase separation was formed in the mixed solutions of them being previously pressurized respectively. These results suggest that the assembling of PEG and Dex, but not assembling of PEG or Dex molecule itself, was induced by the pressurization. It is considered that the assembly of PEG and Dex, whose apparent molecular weight was increased as compared to PEG and Dex molecules, was accumulated in the lower phase and gave rise to this phase separation. Also, this phenomenon was observed for the case of PEG (Mw: 8000) used. DLS measurement of them before/after pressurization was carried out. The particle size was increased by pressurization, and then decreased by heat treatment at 50 degrees, indicating the formation of novel hydrogen bonding assembly. On the other hand, for PEG-Dex 500,000 system, an aqueous two-phase system was formed with light scattering in the lower phase before the pressure treatment and then maintained during pressure processing.

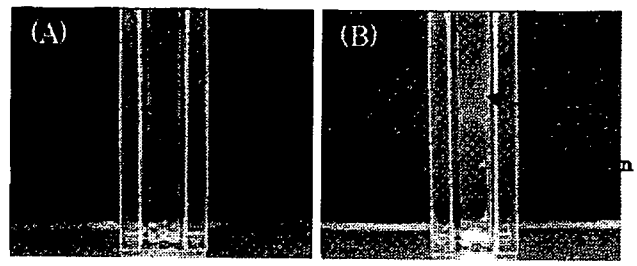

Fig1. Photographs of the mixtures of PEG(6,000) and DEX(60,000~90,000).
(A) without or (B)with ultra high pressure treatment.

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2-P-2

pH応答性ナノ無機粒子を含有する水素結合性ポリマー／DNA複合体の細胞内送達

木村　剛[1]、吉澤　秀和[2]、古薗　勉[3]、藤里　俊哉[3]、岸田　晶夫[1]

[1]東京医科歯科大学　生体材料工学研究所、[2]岡山大学　環境理工学部、[3]国立循環器病センター研究所

我々は、細胞障害性の軽減を目指し、ナノ無機粒子を含有する水素結合性ポリマー／DNA複合体の細胞への遺伝子送達について検討している。複合体は、エンドサイトーシスにおけるpH低下により無機粒子が溶解し、浸透圧変化によりエンドソームが崩壊することで細胞内に取り込まれると考えられる。これまで、ナノHAp粒子／水素結合性ポリマー／DNA複合体の細胞内送達が認められた。本研究では、更なる細胞内送達効率の向上を目指し、種々のpH応答性を有するナノ無機粒子を用いた複合体の細胞内導入について検討した。湿式法にてpH応答性を有するナノ無機粒子を調製し、種々のポリマーとDNAとのハイブリット化を超高圧処理により行い、ナノ無機粒子／水素結合性ポリマー／DNA複合体を得た。蛍光ラベル化DNAを用いた細胞内導入量測定においては、pH応答性の高いナノ無機粒子の場合に高効率な細胞内導入が示され、pH応答性のコントロールによる細胞内導入制御の可能性が示された。

3D11 超高圧誘起ナノ高分子集合体のDDSへの応用

（東医歯大・生材研）○木村剛、（日大・理工）三浦義之、栗田公夫、（岡山大・環境理工）六雄伸悟、吉澤秀和（国循セ研・再生医療）藤里俊哉、（東医歯大・生材研）岸田晶夫

【緒言】

近年、ファンデルワールス力、クーロン力、水素結合など比較的弱い分子間相互作用によって形成される分子集合体に関する研究が活発に行われている。その多くは、精密な分子設計に基づき合成された分子が熱、塩濃度、pHなどの条件が最適化された環境において集合化される。我々は、6000気圧以上の高圧条件下において物質の相互作用にうち水素結合が強調されることに着目し、新たな分子集合体化法として、水素結合性高分子への超高圧印加処理による分子集合体の形成について検討している。本研究では、汎用的な水素結合性高分子を用いて、さまざまな高圧印加条件での高分子集合体形成に関して検討し、DDSへの応用について検討した。

【実験】

水素結合性高分子としては、種々の分子量のPVA、ポリエチレングリコール（PEG）、デキストラン（Dex）を用いた。各水溶液を所定の濃度に調製し、単独あるいは混合した後、高圧印加装置（Dr.chef：（株）神戸製鋼所）を用いて種々の圧力印加条件下にて超高圧印加処理を施した。SEM観察、DLS測定にて高分子集合体の物性解析を行った。また、DDSへの応用として、プラスミドDNAの細胞内送達について検討した。DNAと各高分子の混合溶液に超高圧印加処理を施し、アガロースゲル電気泳動、DLS測定、AFM観察により複合体形成を確認し、複合体の培養細胞への導入について検討した。

【結果・考察】

5w/v%のPEG、Dex水溶液に超高圧印加処理を施した場合、単独溶液では分子集合体形成は確認されなかったが、PEGとDexの混合溶液への超高圧印加処理により、約200nmのPEG/Dex複合体が形成されることが明らかとなった。一方、PVA溶液への超高圧印加処理では、単独溶液にてPVA集合体が得られた。得られる複合体は、PVA濃度、圧力、印加時間により大きく異なり、低濃度、低圧力、短期間の圧力印加にてナノサイズの集合体が得られる傾向があった。上記の複合体は、尿素存在下では得られなかったことから、水素結合を介した集合体であることが示唆された。プラスミドDNAとPVAの混合溶液への超高圧印加処理により、ナノサイズのPVA/DNA複合体が得られることがアガロースゲル電気泳動、DLS測定により明らかとなった。また、蛍光ラベル化DNAを用いて複合体の培養細胞への導入について検討した結果、細胞内での蛍光が観察され、超高圧誘起高分子集合体のDDS担体としての応用が期待された。

【謝辞】

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Application of nano-polymeric assemblies induced by ultra high pressurization for DDS, Tsuyoshi KIMURA[1], Yoshiyuki MIURA[2], Kimio KURITA[2], Shingo MUTSUO[3], Hidekazu Yoshizawa[3], Toshiya Fujisato[4], Akio Kishida[1]. [1] Institute of Biomaterials Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, 101-0062 Tokyo, Tel 03-5280-8029, Fax 03-5280-8028, e-mail kimurat.fm@tmd.ac.jp, [2] Nihon University, [3] Okayama University, [4] National Cardiovascular Center Research Institute

(52) Cellular Delivery of DNA-Polymer Complex Encapsulating Inorganic Nanoparticles Prepared by Ultra High Pressurization

Kimra T., Okada M., Furuzono T., Yoshizawa H., Fujisato T., Kishida A.

Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo, 101-0062 Japan
Dept Bioengineering, National Cardiovascular Center Research Institute, Suita city, Oksaka, Japan
Department of Material and Energy Science, Okayama University, Okayama City, Okayama, Japan
Department of Biomedical Engineering, Osaka Institute of Technology, Asahi-ku, Osaka, Japan

We have developed gene delivery system using DNA complex with non-ionic, water soluble polymers via hydrogen bond through ultra high pressurization (UHP) because the inter-, intra-molecular weak hydrogen bonding interaction was strengthen under high pressure condition. Previously, polyvinyl alcohol (PVA) was used as a model hydrogen bonding polymer, and the PVA/DNA complexes were formed by UHP treatment. Although the PVA/DNA complexes were up-taken by cells, a little enhancement of gene expression was observed using them. Therefore, in this study, to promote the endosomal escape of transferred DNA, we performed the development of PVA/DNA complexes encapsulating inorganic particle, which are dissolved under low pH condition in endosome vesicles and then the rupture of endosome is induced by osmotic shock, using UHP technology. Plasmid DNAs encoding luciferase gene or enhanced green fluorescent protein (EGFP) gene under CMV promoter were used. Nano-scaled inorganic particles having the average diameter of 50–200 nm were synthesized by modified micro-emulsion method. Nano-inorganic particles were dispersed ultrasonically in PVA solution and then mixed with DNA solution. Their mixtures were treated under 10,000 atmospheric pressures at 40°C for 10 min. By SEM observation, the irregular surface of PVA/DNA complexes including inorganic particles was observed, indicating the encapsulation of inorganic particles in PVA/DNA particle. The PVA/DNA complexes encapsulating inorganic particles showed a higher transfection activity. These results indicate the utility of the PVA/DNA complexes encapsulating inorganic particles prepared by UHP method for DNA delivery.

2W09

高圧技術を用いたプラスミドＤＮＡの凝縮操作

東医歯大生材研　○木村剛
日大理工　三浦義之、栗田公夫
岡大環境理工　小野努、吉澤秀和
大工大工　藤里俊哉
東医歯大生材研　岸田晶夫

【緒言】 非ウイルス遺伝子デリバリーにおいては、正電荷物質との静電的相互作用により DNA を凝縮させる手法が主流の一つである。凝縮による DNA の核酸分解酵素耐性の向上、細胞表面との効率良い相互作用により目的の細胞へと送達される。しかしながら、正電荷に由来する細胞障害性が従来から問題とされ、また最近では、強度のＤＮＡ凝縮による転写因子の認識抑制が指摘されている。そこで我々は、静電的相互作用を介さない DNA 凝縮法について高圧技術を用いて検討した。高圧技術は、熱力学パラメータとしての点から圧力下でのタンパク質の構造変化に関する検討がなされている［１］。クーロン力、疎水性相互作用、ファン・デル・ワールス力などの種々の相互作用が複雑に介して構造形成するタンパク質は、加圧によりそれらの相互作用が変化し、構造変化すると考えられている。核酸への高圧印加の影響については、600MPa の静水圧印加によるオリゴ DNA・RNA の B 型から Z 型へのコンフォメーション変化［２］や、圧力の上昇に伴う閉環状プラスミド DNA のスーパーコイル形成の促進［３］などが報告されている。また、圧力上昇に伴うヘアピンリボザイムの活性の減少［４］、あるいは、高圧処理したプラスミド DNA の大腸菌への形質転換効率の向上［５］などと核酸の構造／機能への高圧の影響が検討されている。本研究では、高圧印加によるプラスミド DNA の立体構造変化について詳細に検討し、さらに、真核細胞への遺伝子導入への応用を目的に、高圧印加プラスミド DNA の被転写／翻訳について検討した。

【実験】 DNA としては、T7 プロモーターの下流にルシフェラーゼ遺伝子をコードするプラスミド DNA（pT7-luc）を用いた。高静水圧印加装置（Dr. CHEF；(株)神戸製鋼所）を用いて、種々の温度・圧力・時間にて高静水圧印加処理を行った。処理液を、DLS 測定、CD 測定、アガロースゲル電気泳動、AFM 観察にて構造解析を行った。また、高圧処理による DNA の機能解析として、ウサギ網状赤血球を用いた無細胞系転写・翻訳、核酸分解酵素を用いた分解性試験により検討した。

Pressure-induced condensation of plasmid DNA

Tsuyoshi KIMURA[1], Yoshiyuki MIURA[2], Kimio KURITA[2], Tsutomu ONO[3], Hidekazu YOSHIZAWA[3], Toshiya FUJISATO[4] and Akio KISHIDA[1]. ([1] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-surugadai, Chiyoda-ku, Tokyo, 101-0062, Japan, [2] Nihon University, [3] Osaka Institute of Technology)
Tel: 03-5280-8029, Fax: 03-5280-8028, e-mail: kimurat.fm@tmd.ac.jp

Key Word: condensation / plasmid DNA / high pressure / transfection

Abstract: We investigated the effect of pressure on topology of plasmid DNA in order to develop a novel method condensing plasmid DNA. Plasmid DNA was hydrostatically pressurized at various atmospheres for different time. After pressure removal, the obtained plasmid DNA was analyzed by DLS, CD measurements and AFM observation. The sizes of the super-coiled and relaxed forms of the plasmid DNA were decreased with increasing the strength and period of pressurization, and then detected at approximately 25 and 100 nm, respectively, by DLS measurement. This result indicates the condensation of super-coiled plasmid DNA and the super-coiling of the relaxed plasmid DNA. For AFM observation of the pressurized plasmid DNA, the spherical structure of the super-coiled plasmid DNA was observed. Also, the condensed plasmid DNA was significantly transcribed and translated in cell-free transcription/translation system.

【結果と考察】　図1には、10,000気圧、10分間の高圧印加を施したプラスミドDNAのDLS測定結果を示す。プラスミドDNAでは、約100 nm、600 nmのサイズのピークが検出された。それぞれ、スーパーコイルと開環状プラスミドDNAであると考えられる。一方、高圧印加処理したプラスミドDNAでは、約25 nmと100 nmのサイズのピークが検出された。開環状プラスミドDNAへの高圧印加によるスーパーコイル形成が報告されていることから［3］、後者の約100 nm付近のサイズピークはスーパーコイル状のプラスミドDNAと考えられ、前者はスーパーコイルの凝縮体と考えられる。原子間力顕微鏡観察においては、未処理プラスミドDNAでは、開環状のプラスミドDNAおよび繊維状のスーパーコイルDNAが観察され、高圧印加処理した場合では球形のプラスミドDNAが観察された。以上の結果から、高圧印加によりプラスミドDNAが凝縮され、大気圧に戻した場合でも維持されることが明らかとなった。次に、種々の圧力にて5分間の高圧印加を施した結果、スーパーコイルと開環状プラスミドDNAのいずれの場合も、それらのサイズは圧力の上昇に伴い減少した。また、10,000気圧での異なる時間の施圧において、施圧時間の延長に伴うサイズの減少が示され、これら結果は、スーパーコイルプラスミドDNA凝縮の圧力制御を示していると考えられる。これまで、圧力上昇に伴うオリゴDNAの二重鎖安定性（Tm）の上昇［6］、およびプラスミドDNA形成性の上昇が報告されている［3］。これらは、圧力印加によるDNA二重鎖のねじれの増加に起因すると考えられており、今回のスーパーコイルプラスミドDNAの場合、更なる二重鎖のねじれの増加により凝縮したと考えられる。一方の開環状プラスミドDNAの場合は、高圧印加によりスーパーコイル形成はなされるものの、ニックが入っているため更なる凝縮は困難であると考えている。上述の高圧凝縮プラスミドDNAの機能解析の一つとして、無細胞系転写・翻訳を用いたプラスミドDNAの被転写・翻訳活性について検討した。種々の圧力にて施圧したプラスミドDNAの被転写・翻訳活性は、未処理のプラスミドDNAとほぼ同等、あるいは若干の上昇が示され、非ウイルス遺伝子デリバリーへの応用可能性が示された。

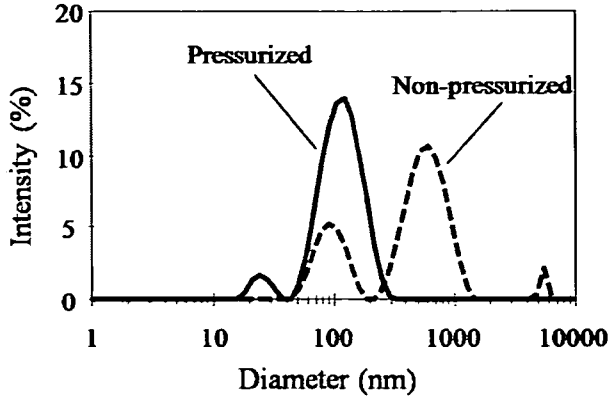

Fig1. DLS measurement of plasmid DNA with/without pressurization at 10,000 atm for 5 min.

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2Pf070

高静水圧誘起 PVA ゲルの特性解析

日大院理工　○三浦義之・栗田公夫　東医歯大生材研　木村剛、
岡山大環境理工　小野努・吉澤秀和　大工大工　藤里俊哉、
東医歯大生材研　岸田晶夫

<緒言>

DMSO／水混合系中での PVA ゲルの作製においては、その過程がゲルの物性に強く影響する。室温下での静置においては、スピノーダル型相分離により誘起される不透明ゲルであり、－20℃以下では、PVA の結晶化が優先され透明なゲルが得られると報告されている。また、凍結 - 融解サイクルの制御により透明かつ機械的強度の優れたゲルが得られると報告されている。しかし、これらの作製法は長時間の静置、あるいは複数回の凍結 - 融解サイクルが必要であり、作製に時間を要する。一方、我々は、水素結合性が強調される高静水圧処理を PVA 水溶液に施すことでゲルが短時間で得られることをこれまでに報告している。本研究では、種々の混合比の DMSO／水溶媒系での PVA ゲルの作製および物性について詳細に検討した。

<実験>

PVA(重合度:1700,鹸化度:99.8%)を、種々の混合比の DMSO/水混合液にて様々な濃度で溶解した。高圧処理装置(Dr.CHEF;(株)神戸製鋼所)を用いて、異なる温度・圧力・時間にて高圧処理を行った。得られた PVA ゲルのマクロ・ミクロ観察、透過度、膨潤度、力学強度測定にて物性解析を行なった。

<結果・考察>

PVA 水溶液、DMSO／水(80／20)混合液への圧力印加(10,000 気圧、10℃、10 分間）により、それぞれ不透明、透明な PVA ゲルが得られた(Fig1)。一方、同温度、同処理時間の放置ではゲルが得られず、圧力処理によるゲル化促進が改めて示された。高圧 PVA ゲル作製における、DMSO／水混合溶媒の混合比、温度、圧力、時間等の圧力条件の影響について詳細に検討を行なった。

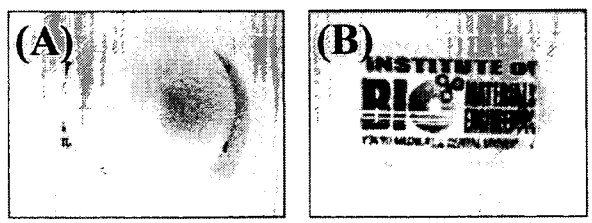

Fig1. Photos of 10%PVA gels using Ultra high pressurization(10,000atm 10degrees 10min). (A):DMSO／W(0／100), (B):(80／20).

まず、DMSO／水の混合比を 20／80、60／40、80／20 とし、10℃にて 10,000 気圧、10 分間の高圧処理を施した。60／40、80／20 ではゲル形成が認められたが、20／80 ではゲル形成されなかった。次に温度の影響について 25℃、40℃で高圧処理したところ、25℃では 60／40 で成形性の良いゲルが、80／20 では脆弱なゲルが形成されたが、20／80 ではゲルが形成されなかった。40℃では、60／40 のみでゲル形成が認められた。さらに処理圧力の検討を行なったところ、60／40、80／20 ではより高圧の条件下にてゲル形成が認められたが、20／80 では 10,0001 気圧の高圧下においてもゲル形成されなかった。以上の結果から、種々の DMSO／水の混合比において、高圧処理温度、圧力に依存的にゲルが形成されることが示された。発表では、種々の条件により得られた PVA ゲルの力学強度、透過性等の物性についても報告する。

Characteristics analysis of PVA hydrogels induced by ultra-high pressurization

Yoshiyuki MIURA[1], Kimio KURITA[1], Tsuyoshi KIMURA[2], Tsutomu Ono[3], Hidekazu YOSHIZAWA[3], Toshiya FUJISAO[4], Akio KISHIDA[2] ([1]Nihon Univ., 1-8-14 Kanda-Surugadai Chiyodaku, Tokyo 101-0062, Japan, [2]Tokyo Medical and Dental Univ., [3]Okayama Univ., [4]Osaka Institute of Technology)
[2]TEL&FAX: +81-3-5280-8029, E-mail: kimurat.fm@tmd.ac.jp

Key Word: ultra high pressure treatment /hydrogel

Abstract: We investigated the effect of pressurizing condition, such as pressurizing strength, time and temperature, on the hydrostatic pressure-induced PVA gels in the mixture of DMSO and water at various ratios of them. When aqueous PVA solution was pressurized at 10,000atm and 10℃ for 10 min, the opaque PVA gel was obtained, whereas the transparent PVA gel was formed in mixtures of DMSO and water (80/20) by the pressurization under the condition described above. The galation was strongly dependent on the mixing ratio of DMSO and water, pressurizing strength and temperature, and then the gel having good formability was obtained under pressure condition of higher pressure and lower temperature.

細胞への遺伝子送達における高圧技術応用

Application of high pressure for gene delivering into cells

○木村剛[1]、南広祐[1]、小野努[2]、吉澤秀和[2]、古薗勉[3]、藤里俊哉[4]、岸田晶夫[1]
1東京医科歯科大学生体材料工学研究所，２．岡山大学環境理工学部，３．国立循環器病センター研究所生体工学部，４．大阪工業大学工学部
○Tsuyoshi Kimura[1], Kwangwoo Nam[1], Tsutomu Ono[2], Hidekazu Yoshizawa[2], Tsutomu Furuzono[3], Toshiya Fujisato[4] and Akio Kishida[1]
1. Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental Univ. 2. Department of Environmental Science, Okayama Univ, 3. Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 4. Division of Biomedical Engineering, Osaka Institute of Technology

（5～10mm あける）

1. 緒言

細胞への外来遺伝子導入による細胞の機能制御法がある。遺伝子導入法としては、正電荷物質との静電的相互作用によりDNAを約数千分の１程度に凝縮した複合体を形成させ、目的の細胞に導入する方法が主流である。得られた複合体は、核酸分解酵素に対する耐性を有し、培養細胞への比較的高い導入効率を示す。しかしながら、正電荷に由来する細胞傷害性と強いDNA凝縮による細胞内での転写・翻訳阻害が問題となる。そこで本研究では、細胞毒性の低減および被転写・翻訳活性の向上を目的に、水素結合を介する複合体の調製について高圧技術を応用し、細胞への遺伝子導入を検討した。また、DNAへの高圧印加による凝縮についても検討した。

2. 実験

水素結合を介する複合体の調製には、水素結合性高分子であるポリビニルアルコール（PVA）、デキストラン（Dex）、ポリエチレングリコール（PEG）を用いた。DNAとしては、蛍光タンパク質遺伝子を組み込んだプラスミドDNA(pEGFP)、ルシフェラーゼ遺伝子を組み込んだプラスミドDNA（pT7-Luc）を用いた。水素結合性高分子とDNAとの混合液を10000気圧の高圧印加を施した。走査型電子顕微鏡（SEM）観察、アガロースゲル電気泳動により複合体の形成を確認した。蛍光ラベル化DNAを用い、複合体の細胞内導入および発現を評価した。DNAへの高圧印加による凝縮は、動的光散乱法（DLS）により検討した。

3. 結果と考察

水素結合性高分子を異なる濃度で調製した。DNA溶液と混合し、超高圧処理（10000気圧、10分間）を施した。PVAを用いた場合、濃度の上昇に伴い透明溶液、白濁溶液、ゲル化と溶液のマクロ変化が観察された。透明溶液では約200nmのナノ粒子が、白濁溶液では約１μmの微粒子がSEMにより観察された。またDexおよびPEGの場合は、若干の粘性の上昇が認められたものの透明溶液のままであった。DNAとの複合体の形成について、アガロースゲル電気泳動により確認した。いずれの水素結合性高分子においても、超高圧処理した場合でのみスメアーなバンドが見られ、複合体が形成されていることが明らかとなった（Fig2）。水素結合阻害剤である尿素の存在下では複合体が形成されず、水素結合を介在した複合体であることが示された。熱融解測定では、DNAおよびPVAとDNAの混合液の超高圧未処理の場合、融解温度の変化は認められなかった（約57℃）が、DNAの超高圧処理した場合は融解温度が上昇した（約62℃）。また、DNA/PVA複合体では、40～50℃付近のブロードな変化と約54℃のシャープな変化が観察され、54℃付近でDNA二重らせんの解離が起こっていると考えられ、40～５０℃にかけての融解温度は複合体の解離であると考えている。核酸分解酵素耐性試験では、複合体の十分な分解耐性が示され、また、無細胞系転写・翻訳試験では、核酸分解酵素存在下で長期間インキュベートした後でも十分な転写・翻訳活性が示された。これらより、長期間の遺伝子導入の可能性が示唆された。さらに、蛍光ラベル化DNAを用いた細胞への導入試験では、細胞内にて十分な蛍光が認められたことから、PVA/DNA複合体の細胞内導入が示された。発表では、高圧凝縮DNAについても詳細に報告する。

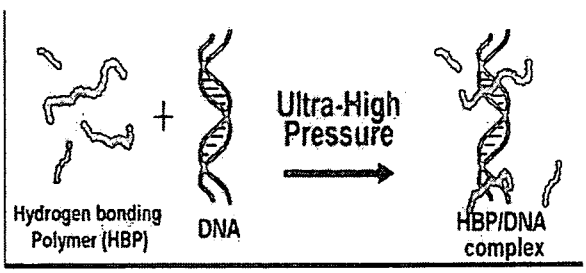

Fig1. Hydrogen bonding polymer/DNA complex via hydrogen bond by ultra-high pressure.

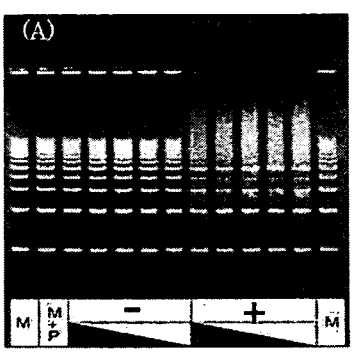

Fig1. Agarose gel electrophoresis of DNA mixtures with PVA treated with ultra-high pressure.

4．謝辞

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