impart durability against collagenase degradation. However, a high percentage of MPC did not immobilize on the collagen gel surface. A longer coupling time or use of larger amount of EDC, NHS, or PMA did not result in an increase in the amount of immobilized PMA. This is believed to be attributable to spatial problems. For immobilization of heparin, low adoption of the polymer with the EDC/NHS coupling reaction was considered a problem. With regard to heparin, it is known that approximately 5.5% immobilization can be achieved [12]. However, activation of the carboxylic group can be triggered at any time point [7]. The coupling reaction continues when EDC/NHS is re-added to collagen gel. Based on this, we developed a novel technique to immobilize the polymer—the "repeat immobilization process."

The repeat immobilization process comprises simply repeating the process of immobilization on the surface of the pre-activated polymer, and a polymer-immobilized collagen gel is thus obtained. The rationale for this process is based on the following: (1) the carboxyl groups can be activated at any time point, and (2) a high percentage of unreacted amine groups are available. Re-activation may promote formation of additional amide bonds, which could not be achieved by a longer coupling time or the use of a higher amount of EDC, NHS, or PMA. In this study, we repeated the immobilization process until a collagen gel with the highest possible number of phospholipid head groups was obtained. Using this gel, we characterized the physical and biological properties of the collagen-polymer hybrid gel. To distinguish the collagen-polymer hybrid gel prepared by the repeat immobilization process from that prepared by time control, we named the former as "collagen-phospholipid polymer hybrid" (a CoPho gel).

2. Experimental methods

2.1. Preparation

2.1.1. Synthesis of PMA

PMA was synthesized according to a previously published method [11,13]. In brief, MPC and methacrylic acid (MA) were dissolved in an ethanol solution. Subsequently, a specific amount of 2,2-azobisisobutyronitrile (AIBN) was added to the ethanol solution. Polymerization was performed in a completely sealed round-bottom flask for 16 h at 60 °C. The solution was precipitated into diethyl ether, freeze-dried, and stored in vacuo until further use. The molar ratio of PMA was MPC:MA = 3:7, and the average molecular weight was 3×10^5.

2.1.2. Preparation of EDC and NHS cross-linked collagen gel (EN gel)

Cross-linked collagen gel was prepared by a previously reported method [11]. Instead of the 0.5 wt% collagen type I solution (pH 3; KOKEN, Tokyo, Japan), 2 wt% collagen type I solution was prepared and used for the film preparation. The collagen solution was dropped onto the polyethylene film and allowed to dry at room temperature. The collagen film (thickness = 56 ± 3 μm) was immersed in a 0.05 M 2-morpholinoethane sulfonic (MES) acid buffer (pH 9.0) (Sigma, St Louis, USA) containing EDC (Kanto Chemicals, Tokyo, Japan) and NHS (Kanto Chemicals). The molar ratio of the constituents was EDC:NHS:-collagen-carboxylic acid groups = 10:10:1. The cross-linking procedure was allowed to proceed at 4 °C for 4 h to produce a cross-linked gel (EN gel). After 24 h, the reaction was terminated by removing the gel from the solution. Subsequently, the gel was first washed with a 4 M aqueous Na_2HPO_4 solution for 2 h to hydrolyze any remaining O-acylisourea groups and subsequently with distilled water over a duration of 3 days to remove traces of salts from the gel.

2.1.3. Preparation of MPC-immobilized gel (MiC gel, MdC gel, and MtC gel; CoPho gel)

MPC-immobilized collagen gel (MiC gel) was prepared using uncross-linked collagen gel (immersed in an alkaline solution at pH 9.0 for 30 min) or EN gel [11]. PMA was added to the MES buffer (pH 9.0) along with EDC and NHS and was activated for 10 min before the uncross-linked collagen or EN gel was immersed. The molar ratios of each chemical was fixed; EDC:NHS:collagen-carboxylic acid groups = 10:10:1. The immobilization of PMA to collagen continued for 48 h at 4 °C. Subsequently, the gel was first washed with 4 M aqueous Na_2HPO_4 solution for 2 h and then with distilled water for 1 day to remove traces of salts and thus prepare a salt-free MiC gel. Fig. 1 illustrates the basic scheme for activation of PMA by EDC and NHS immobilization on collagen. To increase the number of MPC moieties on the collagen-polymer hybrid gel, a MPC-double immobilized collagen (MdC) gel was prepared by immobilizing PMA on the MiC gels by using the same procedure as earlier. To investigate the possibility of further immobilization, we prepared a MPC-triple immobilized collagen (MtC) gel using MdC as the base collagen for PMA immobilization. MiC-0, MdC-0, and MtC-0 were prepared from uncross-linked collagen gels; MiC-1, MdC-1, and

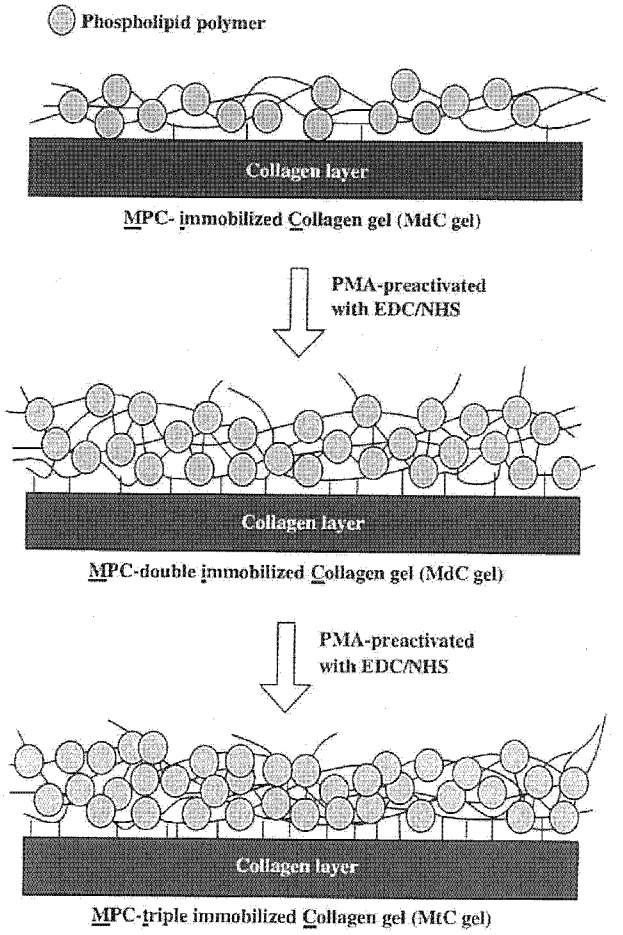

Fig. 1. Schematic diagram of the immobilization process of PMA on collagen.

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MtC-1. from EN gels. The terminology used for the gel samples is listed in Table 1.

2.1.4. Preparation of glutaraldehyde cross-linked collagen gel (G-gel)

Cross-linking collagen gel to glutaraldehyde was performed by a previously reported method [14]. In brief, a 25% glutaraldehyde solution (Merck. Damstadt, Germany) was diluted to 0.5 wt% in phosphate buffer solution (PBS). The collagen film was immersed in the glutaraldehyde/PBS solution and was cross-linked for 3 h at room temperature. After cross-linking, the sample was first rinsed in 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 day to yield a glutaraldehyde cross-linked collagen gel (G-gel). The physical and biological properties of this gel were compared with those of the CoPho gels.

2.2. Characterization

2.2.1. Surface analysis

Surface analysis was executed using X-ray photoelectron spectroscopy (XPS; AXIS-HSi, Shimadzu/KRATOS, Kyoto, Japan) and static contact angle (SCA; ERMA-G1, Tokyo, Japan). The samples that had been cut into small pieces were lyophilized overnight. The chemical composition of the gel surface was determined by the releasing angle of the photoelectrons fixed at 90°. SCA measurement was performed by using a contact angle goniometer (ERMA-G1, Tokyo, Japan) and a Bil-mont syringe. The contact angle of the drop on the surface was measured at room temperature. The SCA experiment was repeated 7 times, and the average was calculated together with the standard deviation.

2.2.2. Determination of the reacted amine group content

The concentration of the primary amine group in tissue samples was determined using a colorimetric assay [15,16]. Three to four milligrams of each sample was prepared. These samples were placed in a 4 wt% aqueous $NaHCO_3$ solution (Kanto Chemicals, Tokyo, Japan) and 2,4,6 trinitrobenzene sulfonic (TNBS) acid. Subsequently, 0.5 wt% aqueous TNBS solution was added (Wako chemicals, Osaka, Japan). The reaction was allowed to proceed for 2 h at 40 °C; the samples were rinsed with saline solution in a vortex mixer to remove unreacted TNBS. Subsequent to freeze-drying the samples overnight, the dry mass was determined. The dry samples were immersed in 2 mL of 6 M aqueous HCl until fully dissolved. The resultant solution was subsequently diluted with distilled water (8 mL) and absorbance was measured at 345 nm (V-560, Jasco, Tokyo, Japan). The concentration of reacted amine groups was calculated using the following equation [16]:

$$[NH_2] = \frac{A \times V}{\varepsilon \times l \times m}, \quad (1)$$

where $[NH_2]$ denotes the reacted amine group content (mol/g collagen gel), ε the molar absorption coefficient of trinitrophenyllysine (1.46×10^4 mL/mmol cm), A the absorbance, V the volume of the solution (mL), l the path length (cm), and m the weight of the sample (mg). The reacted amine group contents of respective collagen gels were all compared with Uc gel.

2.2.3. Swelling test

The swelling test of the samples was executed by cutting the lyophilized gels into small pieces and placing them in a neutral pH aqueous solution at 37 °C. The pH of the aqueous solution was adjusted to 7.4. The gels were gently shaken for 24 h and were measured for assessing the change in weight of the sample. Swelling ratio was calculated in order to define the swelling phenomenon accomplished by water absorption. The experiment was repeated 5 times and the average was calculated along with standard deviation. The following equation was used to calculate the swelling ratio.

$$\text{Swelling ratio, } S(\%) = \frac{W_h - W_d}{W_d} \times 100,$$

where W_h denotes hydrated weight of the gel and W_d the dry weight of the gel.

2.2.4. Fibrinogen adsorption test

Bioresponse was evaluated in terms of protein adsorption by using bovine plasma fibrinogen. The concentration was adjusted to 1 mg/mL. First, the collagen gels were equilibrated by immersing them in PBS. Subsequently, the gels were transferred to the fribrinogen solution, and the solution was incubated for 3 h. After rinsing with PBS, the adsorbed fibrinogen was recovered by dipping the samples in 1 wt% *n*-sodium dodecyl sulfate (SDS) for 60 min [17]. The concentration of recovered fibrinogen was determined at 490 nm by using a Micro BCA kit (Bio-rad, Model 680, Tokyo, Japan).

2.2.5. Cell adhesion test

The interaction between the L929 cells (mouse fibroblast) and the collagen gels was evaluated. The fibroblasts were cultured in Eagle's Minimum Essential Medium (E-MEM, Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA) at 37 °C in 5% CO_2 atmosphere. After treatment with 0.25% trypsin, the cell density was adjusted to 5×10^5 cells/mL and the cells were seeded on the gel surface [18,19]. The collagen gels were sterilized by placing the gels first in an ethanol:water (50:50) solution for 2 h, then in a 70:30 solution for 2 h, and overnight in a 100:0 solution before lyophilization. The lyophilized gels were hydrolyzed with E-MEM for 5 min, and the E-MEM was disposed immediately prior to cell seeding. After 24- and 48-h cycles, the number of adhering cells was measured using UV–vis spectrophotometer (V-560, Jasco, Tokyo, Japan) at 560 nm by lactate dehydrogenase (LDH) assay. All experiments were repeated 3 times and the average was calculated together with the standard deviation.

The morphology of the L929 cells after the 48-h incubation period was observed using scanning electron microscopy (SEM). The cells attached to the samples were rinsed with PBS and fixed with 2.5% glutaraldehyde. Subsequently, the samples were dehydrated for 15 min using an ethanol dilution series (10%, 30%, 50%, 70%, and 90%) before the final dehydration with 100% ethanol. After dehydration, the samples were first dried at room temperature and then in vacuum. In order to avoid deformation of the cells, all samples were fixed onto the glass cover prior to vacuum drying.

Table 1
Terminology of collagen gels used in this study

Terminology		Composition
Uc-gel		Uncross-linked gel (stabilized under pH 9.0)
EN gel		EDC/NHS-cross-linked collagen gel under pH 9.0
CoPho gel	MiC-0	PMA immobilized on Uc-gel under pH 9.0
	MdC-0	PMA immobilized on MiC-0 gel under pH 9.0
	MtC-0	PMA immobilized on MdC-0 gel under pH 9.0
	MiC-1	PMA immobilized on EN gel under pH 9.0
	MdC-1	PMA immobilized on MiC gel under al pH 9.0
	MtC-1	PMA immobilized on MdC gel under pH 9.0

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2.2.6. Cell viability test

The cell viability test was executed using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, USA) assay. The cells were cultured on each test specimen (5000 cells/well) for 48 h and were washed twice with PBS. Subsequently, 200 μl of the MTT solution (0.5 mg/mL in medium, filter sterilized) was added to the culture wells. After incubation for 4 h at 37 °C in a 10% CO_2 atmosphere, the MTT reaction medium was removed and blue formazan was solubilized by the addition of 100 μL dimethylsulfoxide (DMSO). The optical density readings were subsequently performed at 570 nm by using the Micro BCA kit.

2.2.7. Statistical analysis

All the experiments were repeated at least 3 times 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. Statistical analysis was performed using Student's *t*-test with the significant level set as $P<0.05$.

3. Results

Atomic concentration percentage of phosphorus is shown in Table 2. Phosphorus was not detected in the case of Uc gel and EN gel, while phosphorus was detected in the case of CoPho gels. The phosphorus concentration increased for MdC gels, but significant increase in the phosphorus atomic concentration was not shown for MtC gels.

SCA of the respective collagen gels was measured and is illustrated in Fig. 2. The SCA for Uc and EN gels was approximately 70°. SCA decreases as a result of repeating the immobilization process, and was 20° for MtC-0 and -1 gels, thus indicating that the CoPho gel was acquiring a hydrophilic nature.

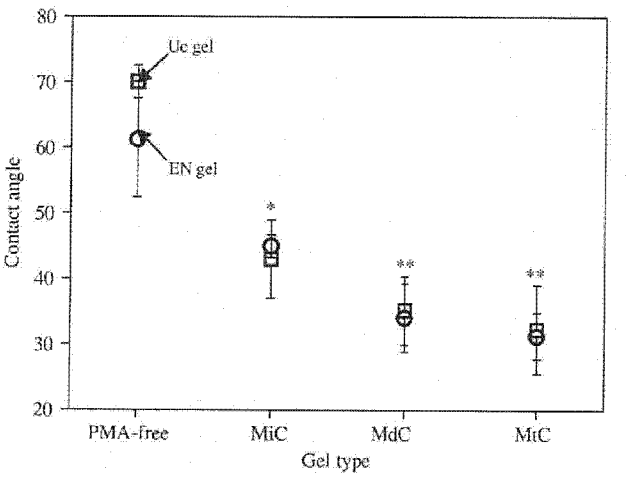
Fig. 3 illustrates the results of the reacted amine group content of the respective collagen gels. It decreases from approximately 60–30% as the PMA is immobilized compared to Uc gel. The lowest reacted amine group content was observed for G-gel, which was approximately 15% of Uc gel.

Fig. 4 illustrates the swelling ratio of the collagen gels under acidic and neutral pH conditions. Uc gel dissolved in acidic pH conditions (pH 2.4) and swelled to approximately 350% in neutral pH conditions (pH 7.4). The swelling ratio decreased rapidly on execution of cross-linking. In terms of the swelling ratio, the two gels did not differ substantially under neutral pH conditions, contrary to the situation under acidic pH conditions. However, a decrease in the swelling ratio was observed; after re-immobilization it decreased from 130% for MiC-1 gel to 95% for MtC-1 gel.

Degradation by collagenase demonstrated that cross-linking decreases the degradation rate of collagen gels (Fig. 5). Uc gels, which completely degraded within 6 h, remained undegraded for 24 h when cross-linked with EDC/NHS alone. Immobilization stabilized the gels against degradation by collagenase.

Fig. 6 illustrates the results of fibrinogen adsorbed on the surface of the collagen gel. It can be clearly seen that fibrinogen adsorption decreased as cross-linking proceeded. The amount of adsorbed fibrinogen further decreases as the MPC polymer is immobilized.

Fig. 7 illustrates the results of the cell adhesion test. Here, we discovered that repeated immobilization of PMA suppressed cell adhesion. Comparison of cell adhesion on completion of 24- and 48-h cycles revealed that the number of adhered cells for in the case of the Uc gel after 48-h cycle had increased by approximately 2 times; the rate of



Uc gel and EN gel $p < 0.01$ vs. * and **
* $p < 0.01$ vs. **

Fig. 2. The static contact angle of the collagen gels. Each value represents the mean ± SD ($n = 5$).

Table 2
Atomic phosphorus concentration of respective collagen gels

Gel type		Atomic phosphorus concentration (%)
Uc gel		0
EN gel		0
CoPho gel	MiC-0	0.21 ± 0.06
	MdC-0	0.36 ± 0.05
	MtC-0	0.37 ± 0.06
	MiC-1	0.18 ± 0.02
	MdC-1	0.37 ± 0.09
	MtC-1	0.36 ± 0.05

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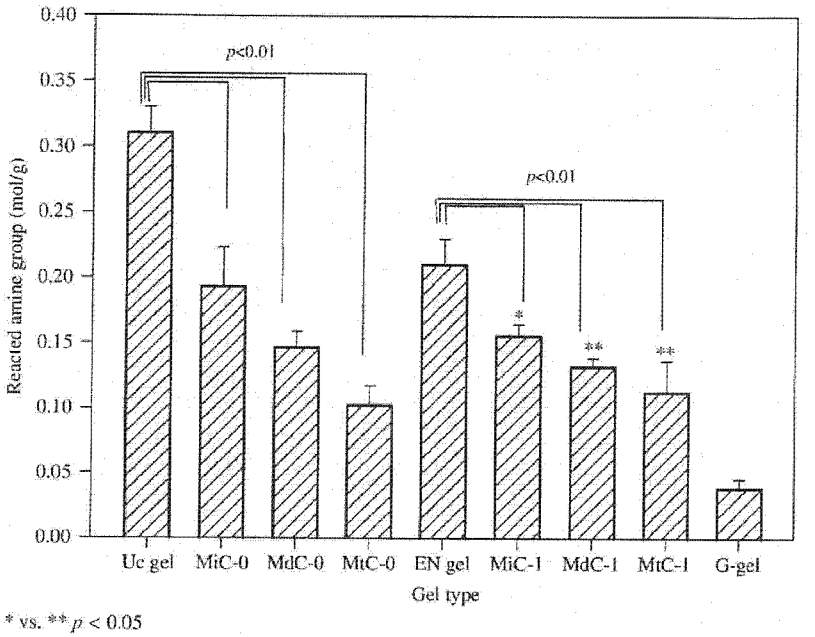

Fig. 3. The percentage of free amine group contents of respective collagen gels. Each value represents the mean ± SD ($n = 5$).

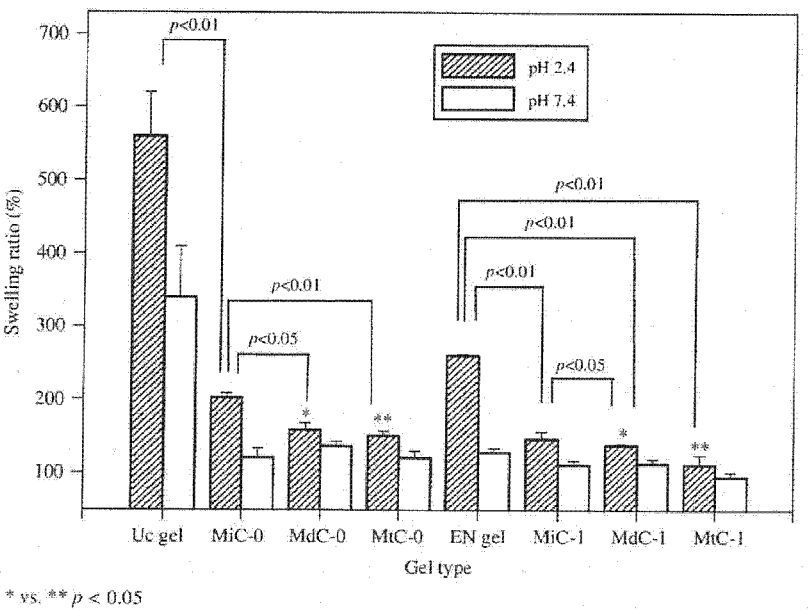

Fig. 4. Swelling ratio of the collagen gels under acidic pH conditions (pH 2.1) (hatched bar) and under neutral pH conditions (pH 7.4) (empty bar). Each value represents the mean ± SD ($n = 5$).

increase decreases as the cross-linking of the collagen gels is proceeded. When assessing the suppression of cell adhesion in terms of intra- and interhelical cross-links, we observed that higher suppression was considerably higher in gels with intra- and interhelical cross-link. MtC-1 gel displayed cell adhesivity that was similar to that of the G-gel.

Cell morphology observed using SEM (Fig. 8) demonstrated that the L929 cells were deformed on the non-MPC surface. On the other hand, the cells remained intact (round) on the CoPho gel surface. An increase in the density of the MPC head group resulted in a decrease in the distribution of L929 cells.

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Fig. 9 illustrates the viability of the L929 cells after 48 h. It reveals that cell viability ranges from 96% to 115% (TCPS as 100%) [20]. Cell viability was approximately 70% for the glutaraldehyde cross-linked collagen gel. Immobilization of PMA did not induce any toxicity.

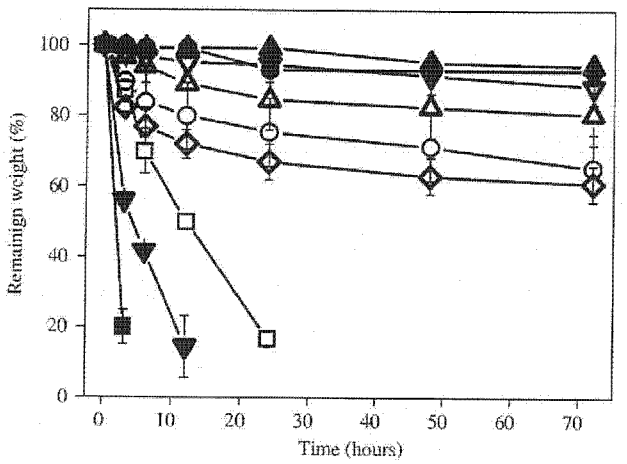


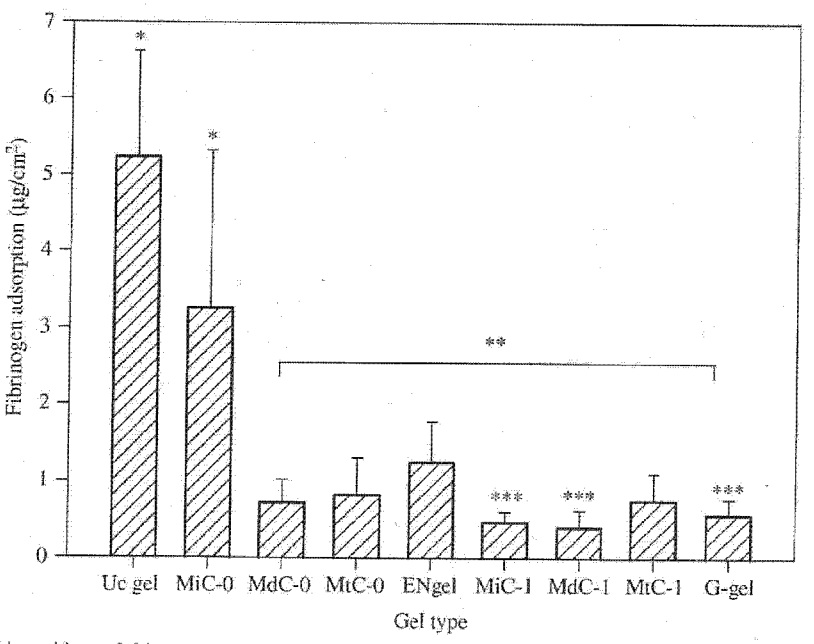
Fig. 5. Degradation of collagen gels by collagenase in Tris–HCl buffer (pH 7.4) at 37 °C. (■) Uc gel, (●) MiC-0 gel, (▲) MdC-0 gel, (▼) MtC-0 gel, (□) EN gel, (○) MiC-1 gel, (△) MdC-1 gel, (▽) MtC-1 gel, and (◇) G-gel. Closed symbols indicate gels without interhelical cross-links while open symbols indicate gels with interhelical cross-links. Each value represents the mean±SD ($n = 5$).

4. Discussion

4.1. Physical properties of the EN and CoPho gels

The collagen gel that was prepared from a 2 wt% aqueous collagen solution differed from that prepared from a 0.5 wt% collagen solution [11]. A considerably thicker film was obtained (≈50 μm), and this film displayed tougher mechanical strength, suppressed swelling, and it slowed collagenase degradation. However, thermodynamic conditions such as shrinkage temperature remained unaltered.

XPS signals displayed a phosphorus peak and a nitrogen peak [$N^+(CH_3)_3$] at 134 and 403.2 eV, respectively; this indicates that PMA was effectively adopted [10,11]. This implies that PMA was successfully immobilized on the surface of the collagen gels. The phosphorus concentration would increase when the MPC is immobilized on the collagen, but did not increase significantly for MtC gels (Table 2). This implies that the immobilization would not occur when the PMA is immobilized for the third time. The increase in the density of the PMA chains is interfering further immobilization process. This can be supplemented by SCA result. The phospholipid head groups on the surface of the collagen gel decreased in the SCA, implying that the surface of the CoPho gel was acquiring a hydrophilic nature (Fig. 2). The hydrophilicty of the CoPho gel was due to the MPC head group, which was located on the surface [18]. The hydrophilic nature of the MPC polymer is thought to be one factor that can suppress



* vs. ** $p < 0.01$
** vs. *** $p < 0.01$

Fig. 6. Fibrinogen adsorption by the collagen gels. Each value represents the mean±SD ($n = 5$).

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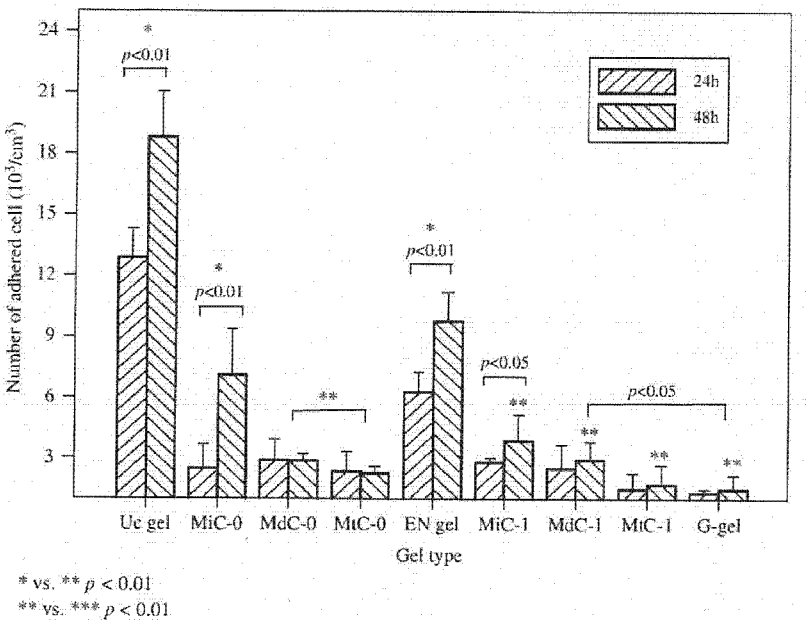

Fig. 7. Cell adhesion property of the respective collagen gels at a seeding density of 5000 cells/cm^2. Each value represents the mean ± SD ($n = 5$).

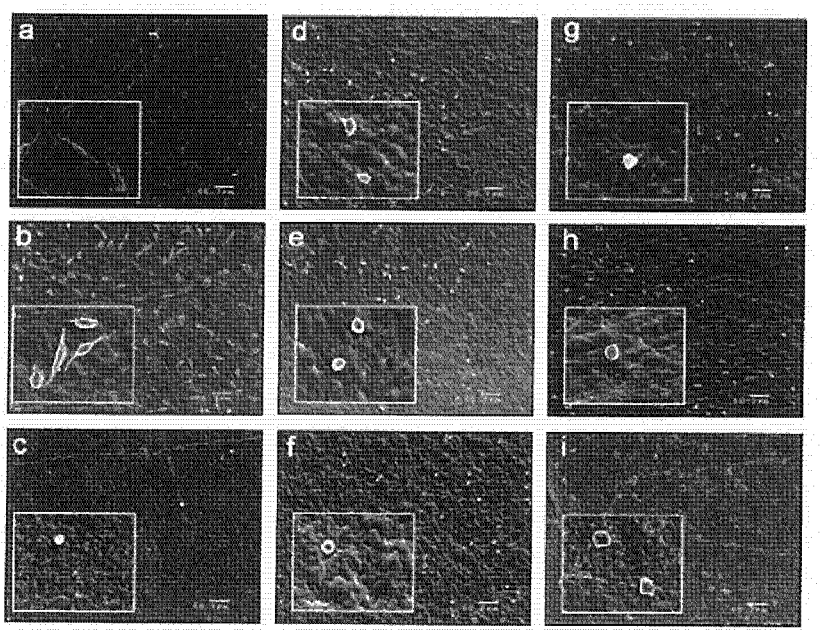

Fig. 8. SEM images of adhered fibroblast cells on respective collagen gels after 48 h of incubation. (a) Uc gel, (b) EN gel, (c) G-gel, (d) MiC-0, (e) MdC-0, (f) MtC-0, (g) MiC-1, (h) MdC-1, and (i) MtC-1 gel. SEM images in the large frame are shown at a magnification of ×150 and the small frame at a magnification of ×1000.

the protein adsorption. It is because the wet condition of the surface is inducing the increase in the mobility of the MPC polymer head group. SCA further decreases as a result of the re-immobilization process, indicating an increase in the density of phospholipid head groups on the surface of the CoPho gels. However, third immobilization process did not decrease the contact angle further.

Did the increase in PMA on the surface of the collagen gel result in a change in the structure of the collagen gels as indicated in Fig. 1? We attempted to characterize the network structure by investigating the reacted amine group content and the swelling ratio (Figs. 3 and 4). Cross-linking collagen gels with EDC/NHS leads to a decrease in the number of reacted amine groups because NH_2 from

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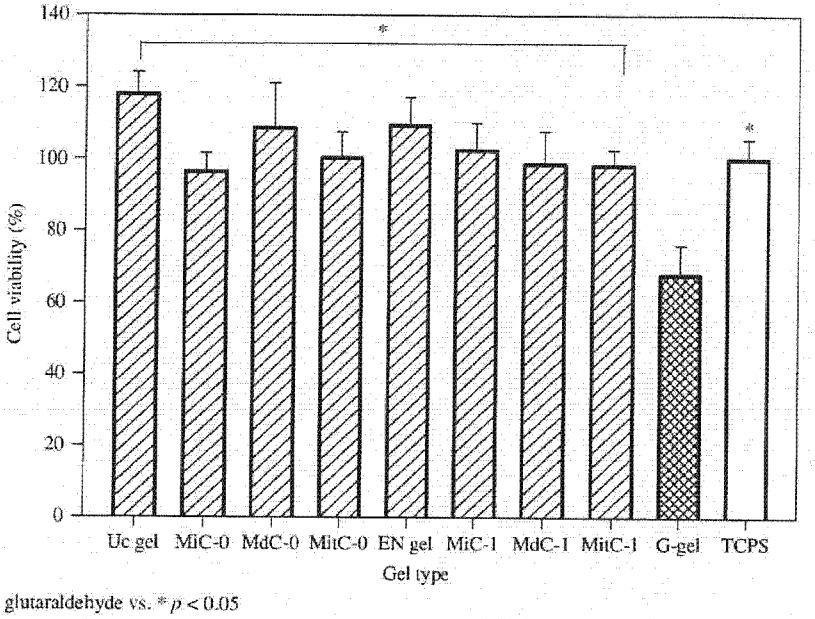

Fig. 9. Cell viability of L929 at 48 h of incubation with at a seeding density of 5000 cells/cm^2 on the respective collagen gels. Each value represents the mean ± SD ($n = 3$).

(hydroxy-)lysine residues is consumed on amide bond formation and NH_3^+ is not formed [7,21]. However, the percentage of the unreacted amine group content was higher than the expected number. Approximately, 60% of the NH_2 remained unreacted compared to Uc gel. Immobilization of PMA on the collagen gel consumed approximately 40% of the amine groups. With regard to PMA immobilization, it is believed that the amine groups may be consumed only on the surface of the collagen gel because PMA cannot penetrate the collagen α-helices [11]. The re-immobilization process decreases the reacted amine group content by up to 40% of Uc gel; immobilization process repeated three times, by up to 30% of Uc gel. The increase in the phosphorus concentration and the decrease in SCA were not observed, but the unreacted amine group content decreased for MtC gels. This implies that the immobilization is stopped, but few intra- and interhelical cross-links have occurred. This decrease is considered high when compared to that of G-gel, which demonstrates approximately 15% of Uc gel. Since the immobilization process occurs only on the surface of collagen gel, lowering the free amine content any further was not possible. An immobilization period of 48 h was the longest period of immobilization that showed a decrease in the number of unreacted amine group, and addition of a higher amount of EDC, NHS, and PMA during the cross-linking process did not cause a significant change in the reacted amine group content (data not shown).

The EN gel is formed by intra- and interhelical cross-links, whereas the CoPho gels are formed by a polymer-helix network. The formation of the cross-link network leads to a decrease in the swelling ratio. In a previous study, we have mentioned that the swelling ratio of the collagen gels is expected to decrease with the progress in cross-linking [11]. The network formed by cross-linking would be dense, and this renders water absorption difficult for the gels. The swelling ratio under acidic and neutral pH conditions varies due to the repulsion force amongst the NH_3^+ and COO^- groups. The swelling ratio is higher for collagen gels immersed in acidic pH condition because they stabilize under neutral pH conditions. With regard to collagen gels prepared with a 0.5 wt% collagen solution, highly acidic pH conditions causes the uncross-linked collagen gels to dissolve [11]; however, none of the collagen gels used in this study dissolved because the α-helices were packed more tightly and were stabilized during gel preparation. Under neutral pH conditions, the collagen film would stabilize by forming a lattice network of fibrils comprising hydrophobic and electrostatic bonds [11,22–24]; hence, the swelling ratio would be less than that observed under acidic pH conditions. The difference in the swelling ratio between the collagen gels with and without inter- and intrahelical cross-links (under acidic pH conditions) is approximately 20–50%. The reacted amine group shows a difference of only 5–10% because stabilization of α-helices by intra- and interhelical cross-links causes a decrease in the swelling ratio. The consumption of carboxyl groups and amine groups is eliminating the site for the protein binding. Arg-Gly-Asp (RGD) site is consumed for the immobilization process, which is making the protein more difficult to adsorbed [25,26]. This would be discussed in Section 4.2.

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Formation of a denser network leads to difficulties in the degradation by collagenase (Fig. 5). Activation of collagenase requires adsorption on the collagen gel surface [11,27]. Subsequently, the collagenase penetrates the collagen gel and begins to cleave the helices [28]. However, a low swelling ratio does not permit collagenase absorption by the CoPho gels. The degradation rate is considerably slower due to an increase in PMA density. A high density of phospholipid head groups is believed to prevent collagenase adsorption on the surface. Eventually, the CoPho gels would be more stable against collagenase.

4.2. Biological properties of EN and CoPho gels

As previously mentioned, the hydrophilicity of the CoPho gel increases with immobilization of PMA due to the presence of phospholipid head group on the CoPho gel. High hydrophilicity is known to be one of the factors that lead to difficulties in protein adsorption [29]. Increase in the density of PMA results in a decrease in the adsorption rate of fibrinogen (Fig. 6). This implies that the immobilized MPC polymer leads to difficulties in the interaction of proteins with the gel surface. In addition to this, the cross-links also decrease protein adsorption. The ε-amino groups from (hydroxy-)lysine residues of collagen are blocked by the cross-linking process [30]. Thus, it is believed that hydrophilicity of the hybrid gel and the blocked ε-amino group renders it difficult for the hybrid gel surface to adsorb fibrinogen. A similar phenomenon was observed during the cell adhesion test (Fig. 7). We observed that repeated immobilization of PMA suppressed cell adhesion. Comparison of cell adhesion after 24- and 48-h cycles revealed that the number of adhered cells in the case of the Uc gel after 48 h cycle had increased by approximately 2 times; this rate of increase would decrease as collagen gels more phospholipids is immobilized. Immobilization of PMA did not induce any toxicity. Decrease in cell attachment on CoPho gels was entirely attributable to the surface property, i.e., the ability to regulate cell adhesion and protein adsorption. These results indicate that the immobilization of the PMA would induce almost the same effect as that of G-gel but without toxicity. As mentioned in Section 4.1, the formation of the cross-linking is eliminating the site for the protein binding. Same affect can be expected for the G-gels. Consumption of RGD for the cross-linking is making the gels to resist against protein adsorption and cell adhesion. Improper cross-linking by glutaraldehyde would induce the high protein adsorption [25]. However, in our case, G-gel showed low protein adsorption and cell adhesion, indicating that the RGD is effectively cross-linked. The formation of the cross-link is bringing the difficulty in the adsorption of proteins.

When assessing suppression of cell adhesion in terms of intra- and interhelical cross-links, we observed that suppression was considerably higher in gels with intra- and interhelical cross-links. The MtC-1 gel displayed cell adhesivity that was similar to that of the G-gel. This implies that intra- and interhelical cross-links also constitute an important parameter in suppression of cell adhesion. This can be reaffirmed by the fact that the number of cells adhered onto the EN gel is less than that in the case of the Uc gel. Much higher affect can be seen for G-gel, but we could not detect high suppression of cell adhesion by EDC/NHS cross-link, indicating that the functional groups still exist largely on the surface. Comparing EN gel and MiC-0 gel, the reacted amine group content is almost the same but the biological property is different. This is due to the difference in the surface property of the EN gel and MiC-0 gel. Investigation of cell morphology revealed that the L929 cells were deformed on the non-MPC surface (Fig. 8). On the other hand, the cells remained intact (round) on the surface of the CoPho gels, indicating a weak interaction between cells and the surface [19]. However, the adsorption of protein and the adhesion of the cell were still higher compared to other materials that used MPC polymer [18,31–33]. Ishihara et al. pointed out that 30 mol% of MPC polymer is required for fibroblast suppression [29]. Repeated immobilization increased the number of phosphorylcholine moieties on the surface of collagen gel surface. However, it is believed that the increase in the number of phospholipids moieties is no longer possible, and no significant decrease was observed in the amount of adsorbed fibrinogen and adhered cells.

5. Conclusion

Repeated immobilization of PMA can increase its immobilization rate, resulting in an increase in the number of MPC head groups; hence, unreacted amine group content and the swelling ratio decreased and the degradation by the collagenase was delayed. The cell morphology remained round indicating a weak interaction between the cells and the gel surface. Thus, the CoPho gel can be used as an alternative collagen-based gel for an implantable biomedical device. Furthermore, we expect that co-immobilization with different polymer-possessing carboxyl groups such as heparin is possible. In the near future, we look forward to reporting on the use of the CoPho gel *in vivo*.

Acknowledgments

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超高圧誘起無機／高分子コンポジットを用いた細胞への遺伝子導入

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<緒言>

遺伝子導入法の一つとして、DNA とリン酸カルシウムの共沈殿によるリン酸カルシウム法があり、簡便かつ低コストで行えることから in vitro にて幅広く利用されている。DNA－リン酸カルシウム共沈殿の生成は pH に依存し、微小な pH 変化、生成時間により得られる共沈殿のサイズが大きく異なるため、遺伝子導入効率の再現性が低い。この問題に対して、赤池らはマグネシウムの添加により結晶成長制御と遺伝子発現効率の向上に成功している[1]。一方近年、無機、有機のそれぞれの特性を併せ持つ無機／有機コンポジットの研究が活発に行われている。遺伝子ベクターにおいては、片岡らが、高分子ミセルの内核においてリン酸カルシウム結晶を生成させ、細胞への遺伝子導入に成功している[2]。ここでは、エンドサイトーシス経路の pH 低下にてリン酸カルシウム結晶が溶解され、浸透圧ショックによるエンドソームの崩壊、核酸分子の細胞質移行が示唆されている。本研究では、無機／高分子コンポジットの調製法として超高圧印加法を導入し、無機物質としてはナノスケールのハイドロキシアパタイト（ナノ HAp）を用いて、無機／高分子コンポジットによる細胞への遺伝子導入について検討した。超高圧印加法では、物質間の水素結合が強調性され、水素結合性高分子と DNA の複合化され、また、複合体にナノ HAp を付加することで上記の機構にてエンドサイトーシスからの遊離が促進されると考えられる。

<実験>

ポリビニルアルコール（PVA）を用いた。HAp は、マイクロエマルジョン法により調整し、種々のスケール（50～400nm）のナノ HAp を得た。遺伝子としては、蛍光タンパク質遺伝子、あるいはルシフェラーゼ遺伝子を有するプラスミド DNA、サケ白子 DNA を用いた。PVA、PEG、DEX を種々の濃度に調製し、0.01～10mg/ml の HAp 溶液、DNA 溶液と様々な割合で混合し、10000 気圧、10 分間加圧した（超高圧処理）。得られた無機／高分子コンポジットの物性を、SEM 観察、電気泳動法、DLS 測定、DSC 測定、CD 測定などの種々の方法にて検討した。細胞として、L929、RAW264、MC3T3、ラット骨髄細胞を用いた。これらの細胞への遺伝子導入を血清存在下で行い、蛍光強度測定、ルシフェラーゼ活性測定により評価した。また、細胞内動態について蛍光ラベル化ＤＮＡを用いて蛍光顕微鏡観察を行った。

<結果・考察>

ナノ HAp を PVA と混合し、超音波処理することで分散性の高いナノ HAp－PVA 溶液が得られ、DNA を添加後、超高圧処理によりナノ HAp／PVA／DNA 複合体が得られた。得られる複合体は、用いるＰＶＡ濃度に依存し、低濃度ではナノ・マイクロ粒子が、高濃度でハイドロゲルが得られた。DNA/PVA/HAp 複合体の SEM 観察により、表面および内部への HAp 含有が確認された。超高圧技術を用いることで、無機／高分子コンポジットを容易に作製できることを示している。蛍光ラベル化 DNA を用いて粒子状の複合体の細胞への取り込みと細胞内動態を検討した。添加後 1 時間では、ナノ HAp を含有しない PVA/DNA 複合体、リン酸カルシウム/DNA 複合体に比べ、ナノ HAp/PVA/DNA 複合体の有意な細胞内導入が示された。24 時間後においてもその導入遺伝子は確認された。また、ナノ HAp/PVA/DNA 複合体の場合に、遺伝子発現の有意な向上が示された。しかし、市販の遺伝子導入剤である Lipofectamine2000 に比して低く、更なる改善が必要である。一方、高濃度のハイドロゲルでは、核酸染色法により DNA の含有が確認された。ハイドロゲル上に数種の培養細胞を播種し、細胞親和性を検討した結果、PVA ゲル上では細胞接着は観察されず、HAp 含有 PVA ゲルにおいて有意な細胞接着が認められた。このハイドロゲルによる遺伝子導入についても報告する。

2Pc185

エンドソーム遊離促進を目指した ナノHAp/PVA/DNA複合体による遺伝子導入

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[緒言] 我々は、超高圧処理により調製したポリビニルアルコール（PVA）とDNAの複合体を用いた細胞への遺伝子導入を検討している。PVA/DNA複合体の細胞内送達は達成されたが、十分な遺伝子発現は認められなかった。そこで、エンドソームの遊離促進を目指し、低pHで溶解されるハイドロキシアパタイトのナノ粒子を含有するナノHAp/PVA/DNA複合体を考案し、遺伝子導入効率の向上が示された。本研究では、更なるエンドソーム遊離促進と遺伝子導入効率の向上を目指し、物性の異なるHApを用いたHAp/PVA/DNA複合体による遺伝子送達について検討した。

[実験] 形状および酸溶解性の異なるHApを用いた。HAp濃度、分散処理条件の最適化を行い、超高圧処理装置（Dr.CHEF；(株)神戸製鋼所）を用いて37℃、10000atmの超高圧処理を施し、ナノHAp/PVA/DNA複合体を得た。得られた複合体の物性を光学・電子顕微鏡観察、DSC測定にて解析した。COS 7細胞への遺伝子送達について、蛍光ラベル化したプラスミドＤＮＡを用いて検討した。

[結果と考察] 超音波処理を20分した後、高濃度DNA溶液に混合した場合に高分散性複合体が得られることが明らかとなった。得られたDNA/PVA/HAp複合体のSEM観察では、表面および内部へのHAp含有が確認された。蛍光ラベル化DNAを用いた細胞内送達では、添加後1時間においてHApを含有しないPVA/DNA複合体、リン酸カルシウム/DNA複合体に比べ、HAp/PVA/DNA複合体の場合に有意な細胞内導入が達成された。24時間後においても導入遺伝子は確認された（図）。これらの結果より、HApによる早期の細胞内送達が示された。

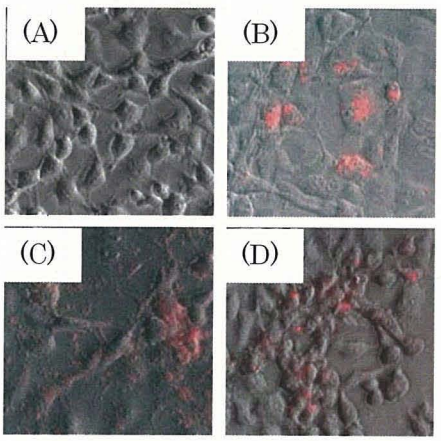

Fig. Gene delivery into COS7 cells using (A) DNA, (B) nano-PVA/DNA (C) nano-HAp/PVA/DNA complex and (D) calcium phosphate/DNA complex.

Gene delivery using nano-HAP/PVA/DNA complexes promoting endosomal escape

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Key word: ultra high pressure / inorganic nano-particles / hydroxyl apatite / hydrogen bond / nano-composite / gene delivery

Abstract: We have researched gene transfection using hydrogen bonding polymer/DNA complex prepared by ultra high pressure technology. In this study, for effective gene transfection, we investigated the preparation of nano-hydroxyl apatite (HAp)/PVA/DNA complex including nano-scaled HAp particles promoting endosomal escape. Gene transfection was carried out using fluorescent labeled plasmid DNA molecules. After 1hour incubation, the effective cellular uptake of HAp/PVA/DNA complexes comparison with PVA/DNA complex and DNA was observed by fluorescent microscope, and then high transfection efficiency was achieved using HAp/PVA/DNA complexes. These results indicate the effective DNA release from endocytosis.

1Pg051

PEG/多糖の水性二相系への超高圧処理による新規構造体の調製

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<緒言>

我々は超高圧技術を用いた新規構造体の創製について研究を行っている。超高圧条件下では、水素結合が強調されることに着目し、これまで、水素結合性高分子であるポリビニルアルコール(PVA)への超高圧印加により、ナノ・ミクロ粒子、ハイドロゲルが形成されることを報告した。本研究では、種々の分子量のPEG/多糖水性二相系の高分子混合液への超高圧処理による、新しい多成分系ポリマー構造体の調製について検討した。

<実験>

種々の分子量のポリエチレングリコール(PEG、Mw:6000、8000)、デキストラン(DEX、Mw:60,000～90,000、50,000)、プルランを用いた。各々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)測定にて構造体の物性解析を行った。

<結果・考察>

各単成分溶液への超高圧処理では、目視による変化が確認できなかった。一方、PEG/DEX混合溶液では、溶液を調製した段階で溶液が青白色を呈した。これは、高分子量のDEXを用いた場合に顕著であった。高分子量のPEGとDEXを混合することにより水性二相分離が形成されることが知られている。今回用いたPEG、DEXは低分子量であることから二相は形成されず、エマルションな状態となったことで散乱が生じ、青白色を呈したと考えている。さらにPEG/DEX混合液への超高圧印加処理では、高分子量のDEXを用いた場合に水性二相を形成し、下相では青白色を呈した（図1）。より高分子量のDEX（Mw=500,000）を用いると、超高圧を印加しない場合でも水性二相を形成するが、処理後では変化は確認できなかった。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観察された。また、50℃におけるDLS測定では粒子径の減少が示されたから、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多成分構造体が形成されたと考えられる。また、PEG／プルラン混合系においても、同様の結果が得られた。PEG/DEX構造体の詳細な解析、および超高圧処理によるその他のPEG/多糖構造体についても報告する。厚生労働省科学研究費ならびに文部科学研究費の補助を受けて行われた。

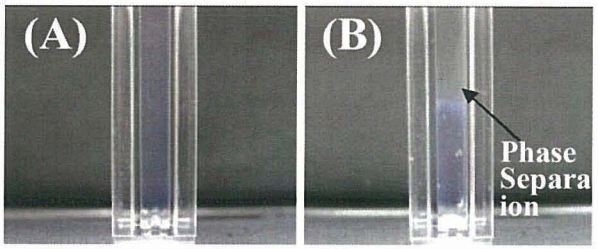

Fig1. Photographs of the mixtures of PEG(6,000) and DEX(60,000~90,000).
(A) without or (B)with ultra high pressure treatment.

Preparation of novel structures by ultra high pressure treatment for aqueous PEG/polysaccharides two-phase system

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Key Word: ultra high pressure treatment / aqueous two-phase system

Abstract: In this study, the preparation of novel structures by ultra high pressure (UHP) treatment for aqueous PEG/polysaccharides two-phase system was investigated. For polyethylene glycol (PEG) or dextran (DEX) solutions, the clear solutions were maintained after UHP treatment. On the other hand, when PEG (Mw: 6,000 or 8,000) were mixed with DEX (Mw:60,000-90,000) or pullulan, the solution with light scattering was obtained. After UHP treatment, aqueous two-phase separation having light scattering in lower phase was obtained for all cases, suggesting that apparent molecular weight was increased by the formation of PEG/ polysaccharides complex. DLS measurement of them before/after UHP treatment was carried out. The particle size was increased by UHP treatment, then decreased by heat treatment at 50 degrees, indicating the formation of novel hydrogen bonding structures.

2Pd184

遺伝子導入能を有する超高圧誘起ナノ無機粒子／高分子／DNA 複合体の調製

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<緒言>

我々は超高圧状態における物質の水素結合の強調性に着目し、種々の水素結合性高分子を用いた超高圧誘起水素結合性高分子集合体による細胞への遺伝子導入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）/DNA 複合体、および、ハイドロキシアパタイト（HAp）を含有する HAp/PVA/DNA 複合体による細胞への遺伝子導入を報告してきた。また、高濃度 PVA への超高圧印加によりハイドロゲルが形成されることから、PVA/DNA ゲルからの DNA の徐放についても取り組んでいる。本研究では、PVA/DNA ゲルに HAp を含有させることで細胞接着性を付与し、接着する細胞への効率的な遺伝子導入について検討した。

<実験>

重合度 1700、鹸化度 99.3%の PVA　を用いた。HAp は、マイクロエマルジョン法により調整し、形態の制御された種々のスケール（50～400nm）の HAp を得た。また、異なる結晶化度の HAp も調整した。遺伝子としては、蛍光タンパク質遺伝子を有する pEGFP プラスミド DNA、サケ白子 DNA を用いた。5、7.5、10w/v%の PVA 水溶液を調製し、0.01～10mg/ml の HAp 溶液、DNA 溶液と種々の割合で混合し、10000 気圧、10 分間加圧した（超高圧処理）。上記複合材の細胞親和性、細胞への遺伝子導入を検討するため、マウス由来の繊維芽細胞（Ｌ９２９）、マウス骨が、ラット骨髄細胞を用いた。ラット骨髄細胞は、ラット大腿骨より採集し、培養シャーレ播種後、接着した細胞を使用した。

<結果・考察>

PVA／HAp／DNA 混合液への超高圧処理により白色のハイドロゲルが得られた。ＰＶＡ溶液の濃度上昇に伴うハイドロゲルの力学的強度の向上が示された。SEM 観察では、ハイドロゲル表面に HAp が観察され、また、DNA 染色法により DNA の含有が確認された。これらの結果は、超高圧技術を用いることで、無機・有機ハイブリッド材料を容易に作製できることを示している。得られたハイドロゲル上に数種の培養細胞を播種し、細胞親和性を検討した。PVA ゲル上では細胞接着は観察されず、HAp 含有 PVA ゲルにおいて有意な細胞接着が認められた。遺伝子導入についても報告する。

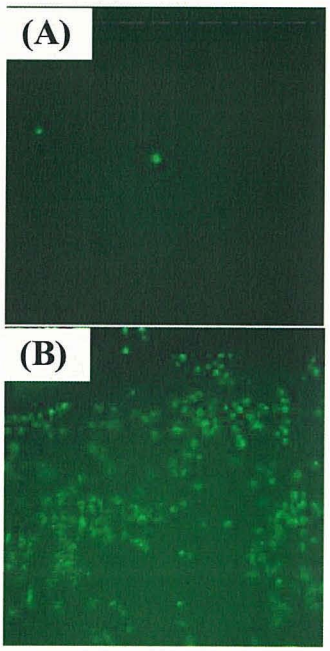

Fig. Adhesion of L929 cells on (A) PVA/DNA and (B) PVA/HAp/DNA hydrogels.

Preparation of nano-inorganic particel/polymer/DNA compsites by ultra high pressure technology for gene transfection

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Key Word: ultra high pressure treatment / hydrogel / transfection / hydroxy apatite

Abstract: We have performed the development of nano-inorgenic particle/polymer/DNA composites by ultra high pressure technology for gene transfection. Nano scaled hydroxy apatite (HAp) was used as nano-inorgenic particle. Polyvinyl alcohol (PVA, 5, 7.5, 10w/v%) aqueous solution mixed with HAp(0.01-10mg/ml) and DNA, then treated by ultra high pressure at 10,000 atm for 10min. HAp/PVA/DNA hydrogel was obtained. By SEM observation, HAp particles on the surface of the hydrogel were observed. By DNA staining, the including of DNA in the hydrogel was confirmed. In order to investigate the cell affinity of the hydrogel, various cell lines were added on the hydrogels. In the case of PVA/DNA hydrogel, almost all of cells were not adhered. On the other hand, the effective cell adhesion was observed on HAp/PVA/DNA hydrogel.

3X08

DNA/RNA構造制御を目指した超高圧印加処理とその応用

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【緒言】

無機・有機科学、医療、食品分野などの幅広い分野で高圧技術が利用されている。例えば、人工ダイヤモンド合成や食品加工・滅菌などで実用されている。また、学術的研究の一つとして、圧力の熱力学パラメータとしての点から圧力印加によるタンパク質の変性に関する検討がなされている。タンパク質は、クーロン力、疎水性相互作用、ファン・デル・ワールス力などの相互作用が複雑に介して様々な構造と機能を有しており、圧力印加による相互作用の変化により変性されると考えられている。高圧下では、一般的に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。そこで我々は、この水素結合性の強調に着目し、水素結合性高分子への圧力印加による水素結合を介する新規構造体の創出について検討している。これまで、水酸基を有する合成高分子であるポリビニルアルコール（ＰＶＡ）への圧力印加により、水素結合を介したナノ粒子、微粒子、ゲルなどの様々な構造体が得られることを報告した。本研究では、天然の水素結合性高分子であるDNAおよびRNAを用い、DNAおよびRNAの高次構造に及ぼす圧力印加の影響とそれらの機能解析について詳細に検討した。DNAとPVAの混合系への超高圧印加によるヘテロ構造体の形成とそれらの細胞への遺伝子導入についてはすでに報告している。

【実験】

DNAとしては、プラスミドDNA、1kbラダーDNAを用いた。それぞれ、TE溶液に溶解し、種々の濃度に調製した。高圧処理装置（Dr.CHEF；(株)神戸製鋼所）を用いて、温度を10、25、37℃、圧力を3,000、6,000、8,000、10,000気圧、時間を1、5、10分の異なる条件にて高圧処理を行った。処理液を、UV測定、Tm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、

Ultra high pressure technology for controlling the structure of DNA/RNA

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Key Word: ultra high pressure / DNA / RNA / structure

Abstract: High pressure technology has been utilized for various fields, such as inorganic, organic science, bio and food science. Pressure is one of thermodynamic parameters, and interactions, such as van der Waals, Coulomb, hydrophobic interaction and hydrogen bond, are varied under pressure condition. Previously, we reported that structuring of polyvinyl alcohol, which is one of the hydrogen bonging polymers, was induced by ultra high pressure treatment. In this study, we investigated the changing of structure of DNA and RNA by ultra high pressure to controlling the functions of them by ultra high pressure for biomedical application It is important to control the structure of DNA and RNA for the function expression.

核酸分解酵素を用いた分解性試験により検討した。

【結果と考察】

図1には、1kb ラダーDNA の Tm 測定の結果を示す。超高圧未処理の DNA では、約57℃付近に、DNA の二重鎖の解離による吸光度の上昇が示された。一方、10,000 気圧、10分間の超高圧処理を施した場合、63℃付近で Tm が観察され、圧力印加による Tm 上昇が示された。また、CD 測定では、1kb ラダーDNA に超高圧印加処理した場合にスペクトルの形状が変化し、280nm、250nm 付近のピークトップのシフトが見られた（図 2)。これらの結果は、超高圧印加による DNA の構造変化を示しており、Tm 測定の結果から新たな水素結合の形成を示唆している。また、核酸分解酵素による分解試験では、超高圧印加処理した DNA での分解耐性の向上が示され、超高圧印加による機能付与と考えられる。

次に、プラスミド DNA に超高圧処理を施した。プラスミド DNA の場合、ラダーDNA に比して顕著な CD スペクトル変化は見られなかった。しかし、10%FBS 存在下にてプラスミド DNA をインキュベートし、その後、無細胞系転写・翻訳システムにて発現するルシフェラーゼ活性を測定した結果、超高圧印加処理を施したプラスミド DNA の場合に、ルシフェラーゼ活性の低下は抑制された（図 3)。これは、構造変化に伴う核酸分解酵素耐性の向上と考えられる。これらの結果について、ラダー DNA の場合は低分子量の DNA の構造変化が検出され、高分子量のプラスミド DNA では十分に検出されなかったと考えている。発表では、上記の結果について詳細に報告する。

【謝辞】

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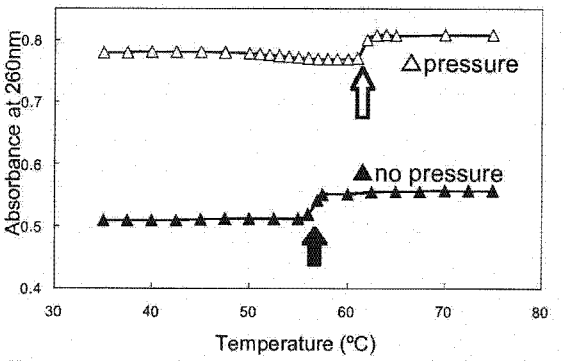

Fig1. Tm measurement of DNA with pressurization at 10,000 atm for 10 min.

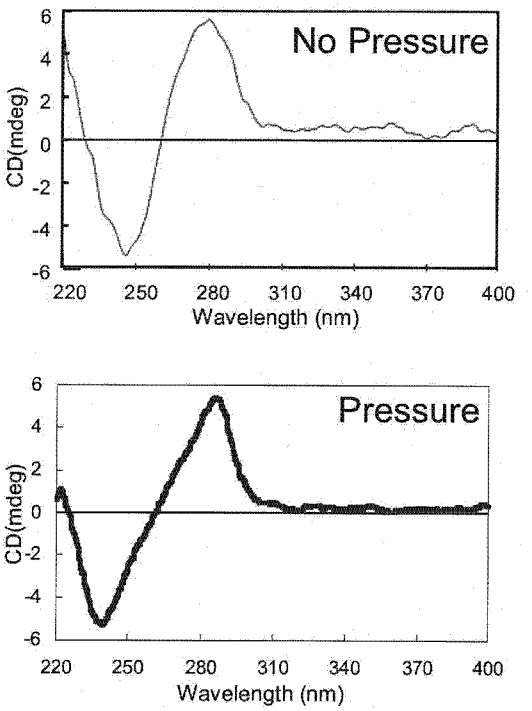

Fig2. CD measurement of DNA with pressurization at 10,000 atm for 10 min.

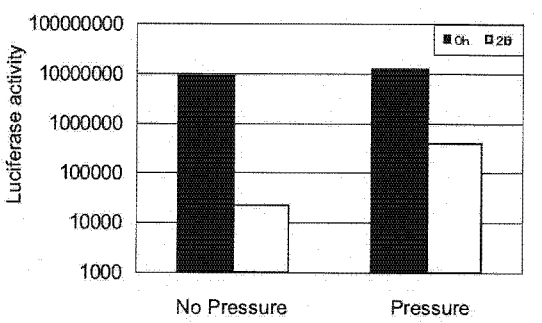

Fig3. Luciferase activity of DNA with pressurization incubated in culture medium with 10% FBS for 0, 20 hours in in vitro transcription/translation system.

PREPARATION OF HYDROGEN BONDING POLYMER STRUCTURES USING ULTRA HIGH PRESSURE TECHNOLOGY AS DRUG CARRIER

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Introduction

Polymeric assembly is a powerful tool for producing functional materials that combine properties and many respond to external conditions (1, 2). Self-assembly of amphiphilic polymers to create nano-micelle (3) or nano-gels (4) has been eagerly studied. They are formed by complex combination of weak interactions, such as hydrophobic interaction, electrostatic interaction and hydrogen bond. To fabricate self-assemble molecules or to break them by controlling these intermolecular forces, alternating concentration and/or temperature is mainly adopted. Here we have focused on pressure, which is one of the fundamental physical parameters as well as concentration and temperature, could also be used for controlling the intermolecular forces to maintain the self-assembled molecules. Pressure processing technology ranging from 1 to 100,000 atmosphere (atm) has been utilized in several fields, such as earth science, material science, food processing, chemistry and biology. In chemistry and biology, the behavior of molecules, such as proteins, artificial peptides and synthetic polymers, has been studied under high pressure condition. It was reported that pressure affected the aggregation properties of elastin, elastin-like peptide (5), poly(N-isopropylamide) (PNIPAM) (6) and poly(N-vinylisobutyamide) (PNVIBA) (7), which exhibit lower critical solution temperatures (LCSTs) derived from their hydrogen bonding and hydrophobic properties.

The strength of pressure at over 6,000 atm is thought as ultra high pressure (UHP). Under UHP condition, the fact that the hydrogen bond between inter/intra molecules is emphasized than electrostatic and hydrophobic interactions is known well (8, 9). Previously, we found that nano-, micro-particles and hydrogels of poly(vinyl alcohol)(PVA) mediated by hydrogen bonding interaction were formed by pressurization (10). Moreover, for drug delivery system, we also reported the formation of hetero-assembling of PVA and DNA using UHP technology and gene delivery into mammalian cells in vitro (11).

In this study, we have demonstrated the preparation of novel assembly via hydrogen bond by UHP treatment using various hydrogen bonding polymers, such as poly(ethylene glycol) (PEG), poly(vinyl pyrrolidone) and dextran. It is well known that aqueous two-phase separation of PEG and dextran having high molecular weights is formed because of their molecular repulsion between them. We hypothesized that the inter/intra molecular hydrogen bonding interaction of PEG and dextran could be induced by UHP processing, in which the hydrogen bonding interaction is strengthen (Figure 1). The assembly of hydrogen bondable polymers was investigated by pressurization under various conditions. The assembly of those and DNA, in which the hydrogen bonding interactions between bases are formed, was also examined by UHP treatment as one of biomedical applications.

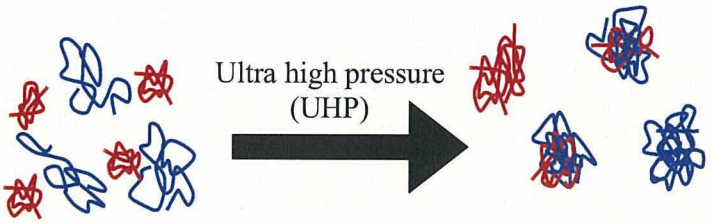

Figure 1. Illustration of polymeric assembly induced by ultra high pressure.

Experimental Part

Poly(ethylene glycol)(PEG), dextran and poly(vinyl pyrrolidone) (PVP) having various molecular weights were used (Table 1). Their chemical structures are shown in Figure 2. They were dissolved in water at concentration of 10 w/v%, respectively and then autoclaved at 121 ˚C for 10 minutes. The PEG solution was mixed with dextran solution and PVP solution at the ratio of 1 to 1, respectively. Their mix solutions were treated under ultra high pressure condition at 10000atm, 25 ˚C for10 min using high pressure machine (Dr.Chef; Kobe steal. Co. Ltd.). The obtained solution was observed by visual observation and the size of the obtained solution was measured using by dynamic light scattering (DLS) measurement. Also, the changing of their sizes during heat treatment was examined by DLS measurement in order to confirm whether the driven force of their assemblies is the hydrogen bonding interaction.

Table 1. Various polymers used.

Polymers	Mw
PEG6	6,000
PEG8	8,000
Dextran60	60,000-90,000
Dextran500	500,000
PVP40	40,000
PVP360	360,000

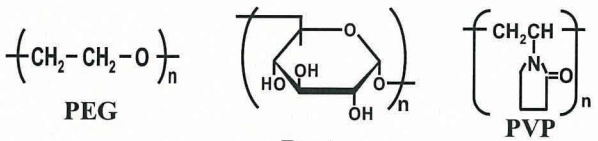

Figure 2 Chemical structures of polymers used

Results

When 10 w/v% solutions of PEG6, PEG8 and PEG35 were pressurized at 10,000 atm, 25 ˚C for10 min, respectively, the solutions were still translucent. Also, in the case of dextran60, dextran100, dextran500, PVP40 and PVP360, there was no change in visual observation of them as they were treated by UHP processing (Figure 3). When the DLS measurement of PEG6 solution and dextran60 solution with/without UHP treatment was carried out at 25 ˚C, the average diameters of PEG and dextran were approximately 4 and 8 nm, respectively, irrespective of UHP treatment. Also, no change in sizes of PEG6 and dextran60 was detected by DLS measurement at 50 ˚C (Table 2). These results indicate that the assemblies of themselves were not induced by UHP treatment. On the other hand, the two-phase separation of mixture solution of PEG6 (5 w/v%) and dextran60 (5 w/v%) was obtained by UHP treatment although the alteration of solution was not observed with mixing of them (Figure 4). The light scattering was observed in lower phase, suggesting that apparent molecular weight was increased by the formation of the assembly of PEG6 and dextran60. In order to confirm the formation of the assembly by UHP treatment, DLS measurement of the mixture solution was performed before and after UHP treatment. The average diameter of their molecules in the mixture solution was increased by UHP

treatment, indicating that the assembling of PEG6 and dextran60 by pressurization (Table 2). The diameter of the assembly was decreased by heat treatment. It suggests that the driving force of the assembly formed by UHP treatment is hydrogen bonding interaction. Similarly, the phase separation of mixture solution of PEG6 (5 w/v%) and PVP40 (5 w/v%) was formed by UHP treatment (Figure 4).

In order to investigate the assembling of PEG and dextran by UHP treatment in detail, PEG and dextran having higher molecular weights were used. The two-phase separation of PEG8 and dextran60 having light scattering in lower phase was induced by UHP treatment, while that of PEG8 and dextran500 was already obtained before UHP treatment. Also, the phase separation was already formed by mixing of PEG6 and dextran500 before UHP treatment. From these results, it was clear that the assembly tended to be formed using dextran having optimal molecular weight. It seems that the assembly could be inhibited due to the intense molecular repulsion between PEG and dextran having higher molecular weight.

When the mixture solution of DNA and PEG6 was pressurized at 10,000 atm for 10 min, the assembling of them was confirmed by agarose gel electrophoretic analysis. The assembly of DNA and dextran60 was also formed by UHP treatment. From these results, they would be utilized as a novel drug carrier.

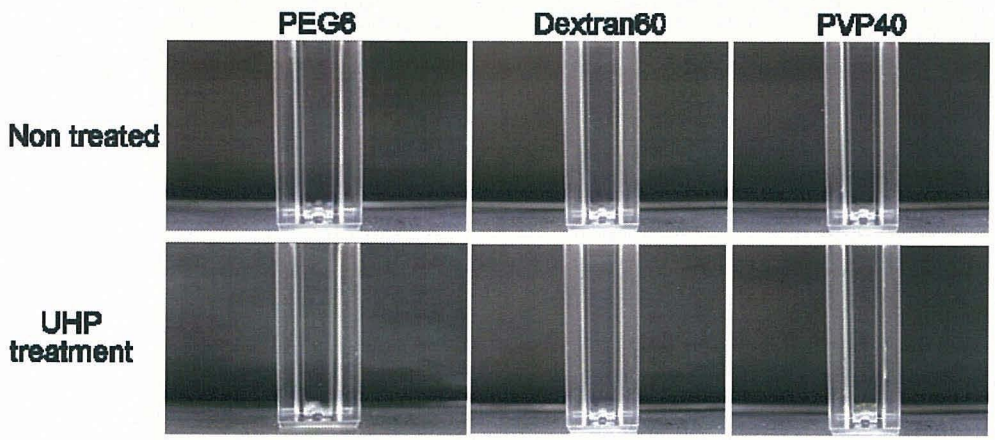

Figure 3. Photographs of polymer solutions of 10 w/v% before and after UHP treatment.

Table 2. DLS measurement of polymer solutions before and after UHP treatment.

Mixtures	Size/nm		
	Non UHP	UHP	UHP
	25℃ (nm)	25℃ (nm)	50℃ (nm)
PEG6	3.6±0.01	3.5±0.04	4.4±0.05
Dextran60	8.1±0.1	7.9±0.04	8.0±0.02
PEG6/Dextran60	112.0±16.0	140.7±7.0	109.5±3.5

* Analysis by cumulant method

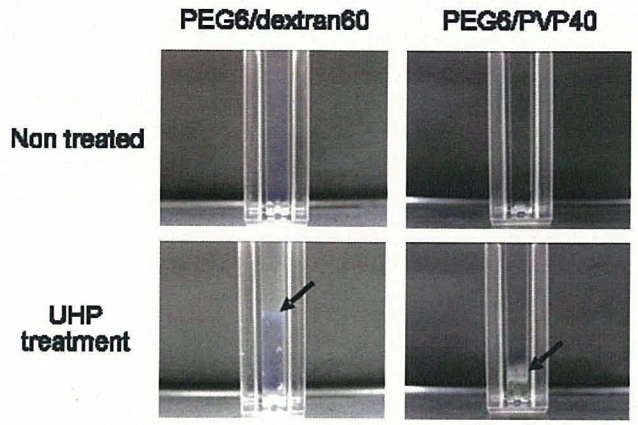

Figure 4. Photographs of mixture solutions of PEG6 and other polymers used before and after UHP treatment.

Discussion

This report described the successful formation of a novel polymeric assembly using UHP technology. DLS measurement confirm the successful assembling of PEG and dextran mediated by hydrogen bonding interaction. Also, PEG and dextran were also assembled with DNA, which has hydrophobic and hydrophilic moieties, by UHP treatment. It is expected that this methodology could be applied to build a structure by the manipulating molecular interactions to develop novel assembly.

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