**Title:** Application of Cross-Linked Salmon Atelocollagen to the Scaffold of Human Periodontal Ligament Cells

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**ABSTRACT**

The purpose of this study was to investigate the application of salmon atelocollagen (SAC) to a scaffold. SAC has a low denaturation temperature and needs to be cross-linked before being used as a scaffold. In the present study, SAC was cross-linked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) or dehydrothermal treatment (DHT). The material properties (degree of cross-linking and solubility in phosphate-buffered saline) of the SAC scaffolds cross-linked by EDC (EDC-SAC) and DHT (DHT-SAC) were evaluated. It was found that EDC-SAC had a high degree of cross-linking and high stability compared with DHT-SAC. Human periodontal ligament (HPDL) cells were cultured in the scaffolds for 2 weeks *in vitro*, and the activities (proliferation rate and alkaline phosphatase [ALP] activity) of HPDL cells cultured in EDC-SAC and DHT-SAC were compared with those cultured in bovine atelocollagen (BAC) scaffolds cross-linked by EDC (EDC-BAC) and DHT (DHT-BAC), respectively. The proliferation rate of HPDL cells cultured in EDC-SAC was equivalent to that in EDC-BAC, and the ALP activity in EDC-SAC was found to be significantly higher than that in EDC-BAC. In the cross-linking by DHT, the cell proliferation rate and the ALP activity in DHT-SAC were lower than those in DHT-BAC. DHT seemed to provide insufficient cross-linking, and DHT-SAC was found to be breakable and

contractile, resulting in less cell activity. In contrast, there was no difference in the thermal stability, porous structure, and cell proliferation rate between EDC-SAC and EDC-BAC. In addition, the collagen helix of EDC-SAC was found to be partially denatured, and this structure resulted in the enhancement of ALP activity of HPDL cells compared with that using EDC-BAC. In conclusion, our results indicate that EDC-SAC could be used as a scaffold for *in vitro* culture.

## INTRODUCTION

Collagen from mammalian sources, primarily bovine skin, has been utilized in foods, cosmetics, and biomaterials, and has the advantage of biodegradability, low toxicity, and immunogenicity. There have therefore been various attempts to use collagen in the medical field as a scaffold for developing artificial organs (1-3). However, the use of bovine collagen has to be reconsidered and limited because of the risks of bovine spongiform encephalopathy (BSE), which is considered to be transmittable to human beings. Recently, there has been interest in nonmammalian collagen sources, primarily fish collagen such as that of shark (4) and salmon (5). It seems that fish collagen has lower risk for transmission of infectious diseases to human beings than bovine collagen (6, 7). We therefore consider that fish collagen could be an alternative to bovine collagen for use as biomaterials.

Fish collagen is more sensitive to heat denaturation than bovine collagen because of its low denaturation temperature, which has made it difficult to use fish collagen as biomaterials. The lower stability is considered to be due to the lower hydroxyproline (Hyp) content of fish collagen compared with bovine collagen (8). The triple helix of collagen is stabilized by intramolecular hydrogen bonds between the OH groups of Hyp. Thus the Hyp content influences the thermal stability. The structure and

physicochemical characteristics of fish collagen have already been investigated (6, 7), but there had been very few studies on the potential for using fish collagen as biomaterials (9). In the present study, we used fish collagen from chum salmon skin, which was discarded as industrial waste, so as to make effective use of natural sources. The salmon atelocollagen (SAC) can be easily extracted at high yields from the salmon skin. We attempted to stabilize SAC by cross-linking methods and to use the cross-linked SAC as a scaffold for *in vitro* cell culture.

Various techniques for stabilizing collagen have been developed and reported. These techniques are divided into chemical treatments and physical treatments. Chemical treatments, using glutaraldehyde (10, 11) and carbodiimide (12, 13), enhance the mechanical properties of collagen. Glutaraldehyde (GA) is one of the most widely used chemical agents; it is known, however, that there are side effects to its use in cross-linking (10), for example, cytotoxicity, enhancement of calcification, and a mild inflammatory response compared with using other reagents. Carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), has been reported to be significantly less cytotoxic than GA because EDC reagents do not remain in the linkage and are simply washed away during the cross-linking process (12). On the other hand, physical cross-linking methods such as ultraviolet (UV) irradiation (14, 15)

and dehydrothermal treatment (DHT) (16, 17) do not introduce any additional chemical units. These methods may therefore be more appropriate than chemical treatments. However, the mechanical properties of materials cross-linked by physical treatments are lower than those cross-linked by chemical treatments, and UV irradiation has been observed to produce insufficient cross-linking in collagen structures, particularly inside large bulk collagen structures. DHT is suitable because cross-links are formed homogenously.

Periodontal disease, which destroys periodontal tissues such as gingival tissue, periodontal ligaments (PDL), and alveolar bone (AB), is known to be a common disease that increases in frequency with age. One of the most widely used regenerative periodontal treatments is the guided tissue regeneration (GTR) technique, which involves the placement of a barrier membrane over the periodontal defects to allow PDL and AB cells to selectively repopulate the isolated place (18, 19). The use of a scaffold impregnated with substances playing a crucial role in cell migration, attachment, proliferation, and differentiation (20) similar to a barrier membrane has been investigated (21, 22). Several different scaffolds have been developed to enhance new periodontal regeneration based on the concept of GTR, and we investigated the application of SAC to such scaffolds.

In our investigation of the application of SAC to a scaffold, we prepared SAC scaffolds cross-linked by EDC and DHT and characterized the material properties of the cross-linked SAC scaffolds. The activities (proliferation rate and alkaline phosphatase [ALP] activity) of human PDL (HPDL) cells cultured in the cross-linked SAC scaffolds were evaluated and compared with those of cells cultured in bovine atelocollagen scaffolds cross-linked by the same methods used for SAC.

## MATERIALS AND METHODS

**Collagen materials** As the fish collagen sample, SAC was used. SAC was isolated according to a previously described method (5). The following procedures were performed at 4 ℃. The fresh skin of chum salmon (*Oncorhynchus keta*) was washed under running water and cut into small pieces. The pieces were then defatted three times with MeOH/$CHCl_3$ and washed with methanol and water. The defatted pieces were suspended in 0.5 M acetic acid. The suspension was centrifuged (10,000×*g*, 30 min) to remove any residues. Pepsin (art. 7185; Merck, Darmstadt, Germany) was then added to the supernatant to a concentration of 30 mg/*l*, and the mixture was stirred for 2 d. Collagen was precipitated by salting-out twice with 5% (w/v) NaCl. The resultant precipitate was dissolved in 0.5 M acetic acid and ultracentrifuged (100,000×*g*, 60 min). The supernatant was dialyzed into distilled water for 5 d and lyophilized. Bovine atelocollagen (BAC) as a control was purchased as a solution from Koken (Tokyo).

**Preparation of a collagen scaffold** Collagen was added to 100 ml of 0.5 M acetic acid to a concentration of 0.5% (w/v) and stirred overnight at 4 ℃. Next, 1 ml of collagen solution was poured into a multiple 24-well plate for tissue culture (Asahi

Techno Glass, Tokyo) and frozen at −70 ℃ for 12 h. The frozen plates were placed into a lyophilizer (FDU-830; EYELA Tokyo Rikakikai, Tokyo) for 24 h.

**Cross-linking methods** The porous scaffolds obtained were immersed in 4 M NaCl aqueous solution containing 1% (w/v) EDC (reagent grade, Dojindo Laboratories, Kumamoto) and placed at 4 ℃. After specific time periods, the cross-linked scaffolds were washed three times with diluted water and then lyophilized again.

DHT was achieved by placing the porous scaffolds in a vacuum oven (VOS-450SD; EYELA Tokyo Rikakikai, Tokyo) and evacuating air above 76 mmHg. Thirty minutes later, the oven temperature was increased to 110 ℃ and maintained for a specific period. The oven temperature was then decreased to room temperature prior to releasing the vacuum.

**Determination of the degree of cross-linking** The degree of cross-linking was determined using 2,4,6-trinitro-benzensulfonic acid (TNBS, analytical grade; Wako Pure Chemical Industries, Osaka) according to a previously described method (23). To a sample of 3-5 mg of the cross-linked scaffold, 1 ml of 4% (w/v) $NaHCO_3$ solution (pH 8.5) and 1 ml of freshly prepared 0.5% (w/v) TNBS solution in distilled water was

added. After allowing the reaction to take place for 2 h at 40 ℃, 2 ml of 6 N HCl was added, and the temperature was raised to 60 ℃. Solubilization of collagen was achieved within 90 min. The resulting solution was diluted with 4 ml of deionized water, and the absorbance was measured at 345 nm with spectrophotometer (U-2001; Hitachi, Tokyo). The degree of cross-linking was calculated as follows:

$$\text{Degree of cross-linking (\%)} = 1 - (\text{Absorbance}_s / \text{mass}_s) / (\text{Absorbance}_n / \text{mass}_n) \quad (1)$$

where the subscripts s and n denote the sample and non-cross-linked collagen, respectively.

**Determination of the solubility in phosphate-buffered saline (PBS)** The solubility of the scaffolds was determined by swelling them in PBS (pH 7.4) at 37 ℃ and measuring the protein concentrations of the supernatant (24). Known weights of scaffolds were placed in PBS for 1, 3, 7, and 14 d at 37 ℃. The supernatants were collected, and their protein contents were measured as described below. The quantity of dissolved collagen was determined by colorimetric assay using a bicinchoninic acid protein assay kit (Sigma Aldrich Japan KK., Tokyo). A 4% copper (II) sulfate

pentahydrate solution was mixed with an excess of bicinchoninic acid at a final ratio of 1 : 50 (v/v), and 100 μl of the supernatants was added to 1 ml of assay solution. The solutions were incubated at 37 ℃ for 30 min, then cooled to room temperature. The absorbance was measured at 562 nm with a spectrophotometer. The solubility was calculated as follows:

$$\text{Solubility (\%)} = 1 - (\text{protein content}_s) / (\text{protein content}_n) \quad (2)$$

where the subscripts s and n denote the sample and non-cross-linked collagen, respectively.

**Scanning electron microscopy (SEM) observation** Cross-linked scaffolds were dried using a critical point dryer (HCP-2; Hitachi Koki, Tokyo). Subsequently, the samples were sputter-coated with gold (E1010/E1020; Hitachi Science Systems, Tokyo) and then observed with a scanning electron microscope (S-403; Hitachi, Tokyo). Pore size was calculated as the mean diameters of the scaffold pores. At least 20 pores were assessed from three different areas of the same sample.

**Cell culture method** HPDL cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies, Rockville, MD, USA) at 37 ℃ in air containing 5% $CO_2$. The culture medium was changed every 3 d. At semiconfluence, HPDL cells were subcultured in the same medium. The cells less than passage 15 were used for the cell activity assay.

Cross-linked scaffolds were sterilized using UV irradiation for 20 min. Then, HPDL cells were seeded into cross-linked scaffolds using capillary action at a density of $5 \times 10^4$ cells/$cm^3$ and cultured in a multiple 24-well plate for tissue culture (Asahi Techno Glass, Tokyo). The medium was changed every 3 d. After specific time periods, the activities (proliferation rate and ALP activity) of HPDL cells were analyzed as described below.

**Cell proliferation rate assay** It was difficult to directly determine the cell number in the scaffolds because of their resistance to bacterial collagenase digestion. Therefore, the cell number within the scaffolds was analyzed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetr azolium, inner salt] assay (CellTiter 96 $AQ_{ueous}$ Non-Radioactive Cell Proliferation

Assay; Promega, Tokyo), which measures relative cell viability. The cultured scaffolds were transferred into new wells, and 1 ml of MTS (5 mg/ml) was added. After incubation for 60 min, the absorbance was measured at 490 nm using a microplate reader (Spectrafluor Plus; TECAN Japan, Tokyo). The calibration curve of the MTS absorbance was made using the cell suspension at various cell concentrations. It has been found that the absorbance and cell concentrations show a linear relationship (data not shown). Data was expressed as cell number ($\times 10^5$ cells/cm$^2$).

**ALP activity assay** For the differentiated cell function of HPDL cells, the ALP activity was investigated (25). The scaffolds were washed three times with PBS and 500 μl of lysis buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM HEPES) was added. The lysates were sonicated for 15 s and centrifuged (2000×*g*). Then 100 μl of 0.02 M $MgCl_2$ in 0.1 M Tris-HCl (pH 8.8) and 4 mg/ml *p*-nitrophenylphosphate (Sigma Aldrich Japan KK; Tokyo) solution in 0.2 M Tris were mixed and preincubated at 37 ℃. Then 100 μl of cell lysates was added to the mixture and incubated at 37 ℃ for 30 min. The absorbance of liberated *p*-nitrophenol was measured at 405 nm with a spectrophotometer. The ALP activity was normalized by the cell number.

**Determination of the collagen helix structure by FT-IR** The collagen structure was estimated by measuring the content of the collagen helix *H* (%) with Fourier transform-infrared spectroscopy (FT-IR 350; JASCO, Tokyo) according to a previously described method (26). The *H* (%) was estimated from the ratio of two mid-infrared bands *R* (= $A_{1235}$ / $A_{1450}$). The calibration curve of the *H* was made by the BAC mixed with the denatured BAC at concentrations of 0, 25, 50, 75, and 100% (v/v). The denatured collagen was prepared by heating BAC at 60 ℃ for 30 min.

**Determination of the effects of denatured collagen on ALP activity** The denatured BAC was coated on the multiple 24-well plate, and HPDL cells were seeded on the plates at a density of $5 \times 10^3$ cells/cm$^2$. After cultivation for 14 d, the ALP activity of HPDL cells was measured as described above. As a control, BAC-coated plates were used.

**Statistical analysis** Experiments were run in triplicate per sample. All data are expressed as means ± the standard deviation (SD) for n = 3. The single-factor analysis of variance (ANOVA) technique was used to assess the statistical significance of the results ($P < 0.05$).

## RESULTS AND DISCUSSION

**Degree of cross-linking of cross-linked SAC scaffolds** SAC has a low denaturation temperature (19 ℃) (5) and needs to be cross-linked before being used as a scaffold. In the present study, SAC was cross-linked by EDC and DHT. The degrees of cross-linking of SAC scaffolds cross-linked by EDC (EDC-SAC) and by DHT (DHT-SAC) are shown in Fig. 1. DHT-SAC resulted in a low degree of cross-linking compared with EDC-SAC. The degree of cross-linking of EDC-SAC leveled off after 48 h to approximately 40%, whereas that of DHT-SAC was only 10.1% at 3 d. The formation of cross-links by DHT was based on the condensation reactions between adjacent amino acid side chains (16, 17). It seemed that the number of cross-linking sites was few because the cross-linking sites were restricted to adjacent functional groups in the condensation reaction. On the other hand, EDC can react with other polypeptide chains and form a number of intramolecular and intermolecular cross-linking sites, which may explain why the degree of cross-linking was found to be different between EDC-SAC and DHT-SAC.

**Solubility in PBS of cross-linked SAC scaffolds** The thermal stabilities of EDC-SAC and DHT-SAC, cross-linked for 48 h and 3 d, respectively, were assessed by

measuring the amount of collagen dissolved in PBS at 37 ℃. The solubility into PBS of EDC-SAC was less than 3% at 14 d, whereas that of DHT-SAC was 17.0% and 36.9% at 7 and 14 d, respectively (Fig. 2). In addition, the contraction of scaffolds was observed only in DHT-SAC after incubation, with the diameter changing from 10 mm to 5 mm. Figure 1 shows that the degree of cross-linking of DHT-SAC was low. It is also known that DHT generates partial fragmentation of the collagen peptide chains and the contraction of matrices (5), which may result in low stability of DHT-SAC. In contrast, EDC-SAC was stable and not contractile after incubation for 14 d because of its high degree of cross-linking (Fig. 1). Dissolved collagen was not detected with BAC cross-linked by EDC (EDC-BAC) and DHT (DHT-BAC) (data not shown).

**SEM observations of EDC-SAC and EDC-BAC** It has been reported that the pore size of the scaffolds affects both material properties (enzymatic degradation and mechanical properties) (27) and cell activity (proliferation and differentiation) (28). SEM images of EDC-SAC and EDC-BAC are shown in Fig. 3. The mean pore sizes of EDC-SAC and EDC-BAC were 41.2 ± 8.5 μm and 40.9 ± 13.4 μm, respectively. SEM observations indicated that there was no difference in the porous structure between EDC-SAC and EDC-BAC.

**Proliferation rate of HPDL cells** To investigate the applications of EDC-SAC and DHT-SAC to a scaffold, HPDL cells were cultured in the scaffolds for 2 weeks *in vitro* and the activities (proliferation rate and ALP activity) of HPDL cells cultured in EDC-SAC and DHT-SAC were compared with those cultured in EDC-BAC and DHT-BAC, respectively. The number of HPDL cells cultured in the scaffolds was measured by MTS assay. Figure 4A shows that the proliferation rate of HPDL cells cultured in EDC-SAC was equivalent to that in EDC-BAC. There was no difference in the thermal stability (Fig. 2) and the porous structure between EDC-SAC and EDC-BAC (Fig. 3). In addition, EDC reagents did not remain in the linkage because they were not incorporated into the cross-linked structure and were simply washed out after the cross-linking process in contrast to other cross-linkers, resulting in less cytotoxicity (12). These results show that the cell proliferation rate using EDC-SAC was the same as that using EDC-BAC. Although DHT is a nontoxic treatment introducing no additional chemical units, the proliferation rate of HPDL cells cultured in DHT-SAC was lower than that in DHT-BAC (Fig. 4B). Figure 2 shows that the dissolution rate of DHT-SAC gradually increases. It therefore appears that DHT-SAC becomes unable to function as a scaffold during cultivation.

**ALP activity of HPDL cells** ALP activity, one of the differentiated cell functions of HPDL cells (29), was measured. ALP activity is one of the most useful markers of osteoblasts, and it is known that ALP increases in the bone formation process, particularly during mineralization. Results show that the ALP activity of HPDL cells increased at 14 d and that there was a difference in activity between SAC and BAC (Fig. 5). In the cross-linking by EDC, the ALP activity of HPDL cells cultured in EDC-SAC was significantly higher than that in EDC-BAC at 14 d, indicating that EDC-SAC had a positive effect on cell differentiation and enhanced the ALP activity of HPDL cells. In the cross-linking by DHT, the ALP activity of HPDL cells cultured in DHT-SAC was lower than that in DHT-BAC. The growth of HPDL cells cultured in DHT-SAC was lower than that in DHT-BAC (Fig. 4B). This low growth resulted in less differentiation of HPDL cells and low ALP activity. EDC-SAC could be used as scaffolds for HPDL cells, resulting in the same proliferation rate and enhancement of ALP activity as those using EDC-BAC.

Enhancement of the ALP activity in EDC-SAC seemed to be due to the difference in the collagen helix structure between EDC-SAC and EDC-BAC because there was no difference in the porous structure (Fig. 3) between the scaffolds and no contraction of

the scaffolds. The collagen helix structures of EDC-SAC and EDC-BAC were estimated by measuring the content of collagen helix *H* (%) by Fourier transform-infrared spectroscopy. The results show that the *H* of EDC-SAC was lower than that of EDC-BAC (Fig. 6), suggesting that the collagen helix of EDC-SAC was partially denatured. It has been reported that SAC sponges cross-linked by DHT are partially denatured compared with BAC sponges (5), and our results were in agreement with that finding. Then, assuming that the denatured collagen enhanced the ALP activity, the effects of denatured collagen on the ALP activity were investigated. The results showed that, compared with native collagen, the denatured collagen enhanced the ALP activity of HPDL cells after cultivation for 14 d (Fig. 7). It has been reported that denatured collagen shows better tissue regeneration than native collagen (30). These results suggest that the partially denatured structure of EDC-SAC enhanced the ALP activity of HPDL cells. The partially denatured structure seems to be an attractive characteristic of SAC, enhancing the cell activity.

The results of the present study demonstrate the application of cross-linked SAC to a scaffold, based on our evaluation of the material properties of the scaffolds and the activities of HPDL cells. DHT seems to provide insufficient cross-linking and DHT-SAC was observed to be breakable and contractile, resulting in less cell activity. In

contrast, there was no difference in the thermal stability, porous structure, and cell proliferation rate between EDC-SAC and EDC-BAC. In addition, the collagen helix of EDC-SAC was partially denatured, and this structure resulted in the enhancement of the ALP activity of HPDL cells compared with that using EDC-BAC. Our results indicate that EDC-SAC could be used as a scaffold for *in vitro* culture.

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**Figure captions**

FIG. 1. Degrees of cross-linking (%) of EDC-SAC (circles) and DHT-SAC (squares), expressed as the percentage of primary amine group content lost after cross-linking. The bar represents the SD (n = 3).

FIG. 2. Solubilities (%) of EDC-SAC (circles) and DHT-SAC (squares) in PBS at 37℃. The bar represents the SD (n = 3).

FIG. 3. SEM images of (A) EDC-SAC and (B) EDC-BAC.

FIG. 4. Proliferation rates of HPDL cells cultured in the scaffolds cross-linked by (A) EDC and (B) DHT, expressed as the cell number calculated from MTS assay results. Circles represent the SAC scaffolds, squares the BAC scaffolds. The bar represents the SD (n = 3).

FIG. 5. ALP activities of HPDL cells cultured in the scaffolds cross-linked by (A) EDC and (B) DHT. Closed column represents the SAC scaffolds, open column the BAC scaffolds. The bar represents the SD (n = 3). Asterisks represent statistical significance with $P < 0.05$.

FIG. 6. Contents of collagen helix *H* (%) of EDC-SAC and EDC-BAC. The bar represents the SD (n = 3).

FIG. 7. ALP activities of HPDL cells cultured on plates coated with BAC (closed

column) or denatured BAC (open column). The bar represents the SD (n = 3). Asterisks represent statistical significance with $P < 0.05$.

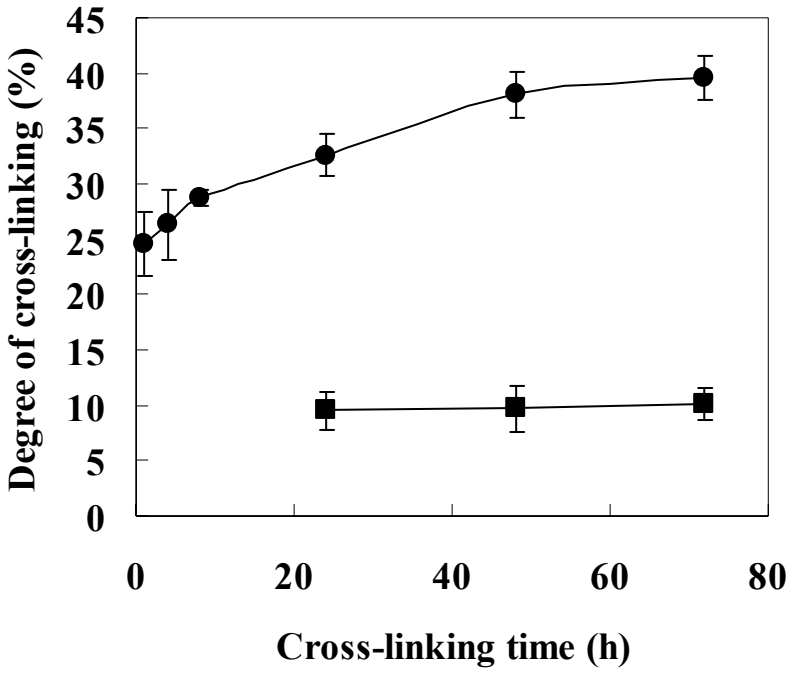

Degree of cross-linking (%)
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
Cross-linking time (h)

Fig.1

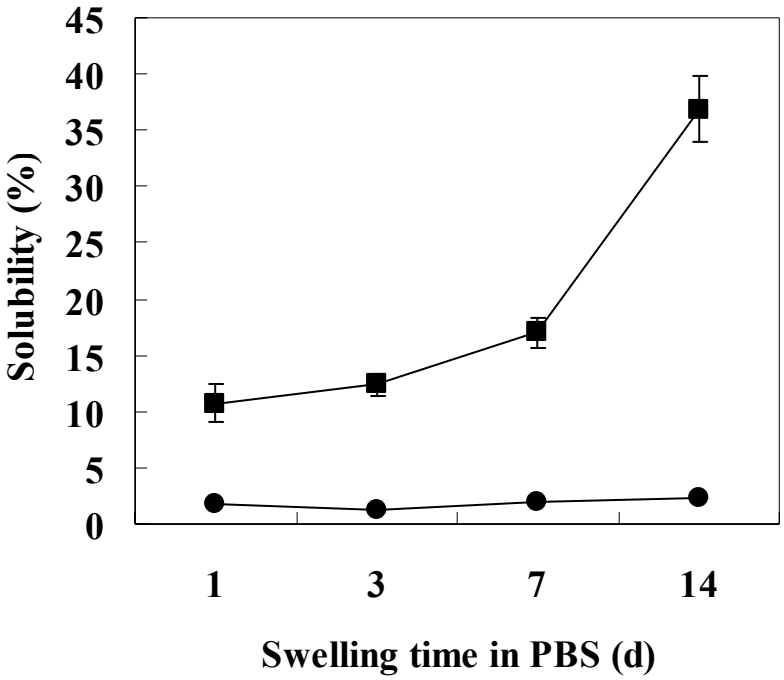
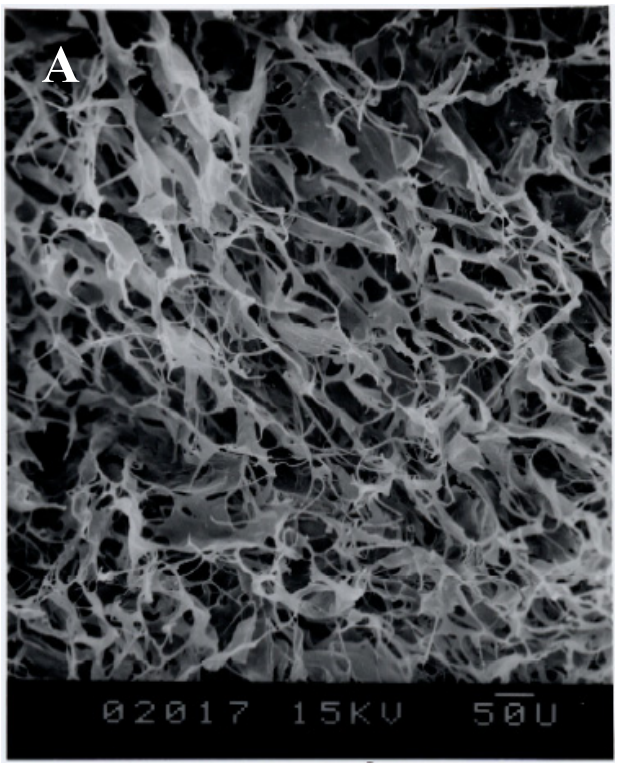

45
40
35
30
25
20
15
10
5
0
Solubility (%)
1
3
7
14
Swelling time in PBS (d)

Fig.2



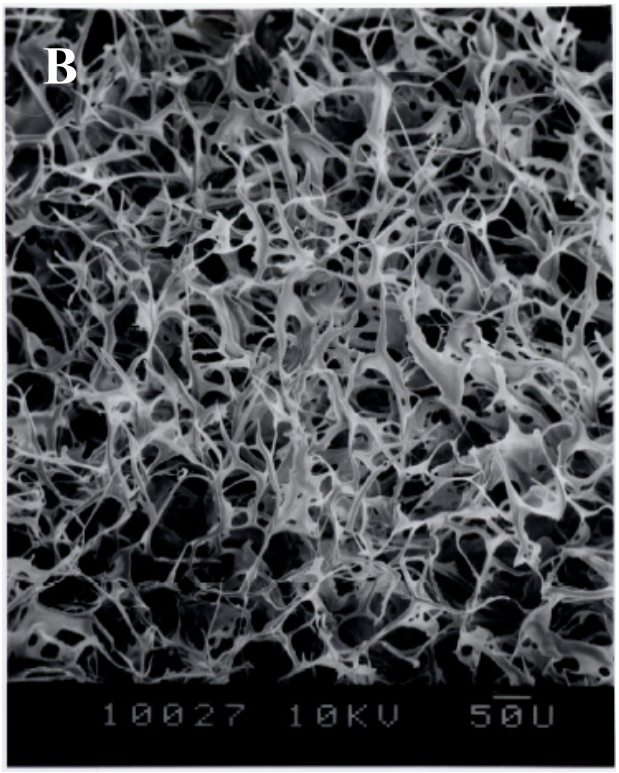
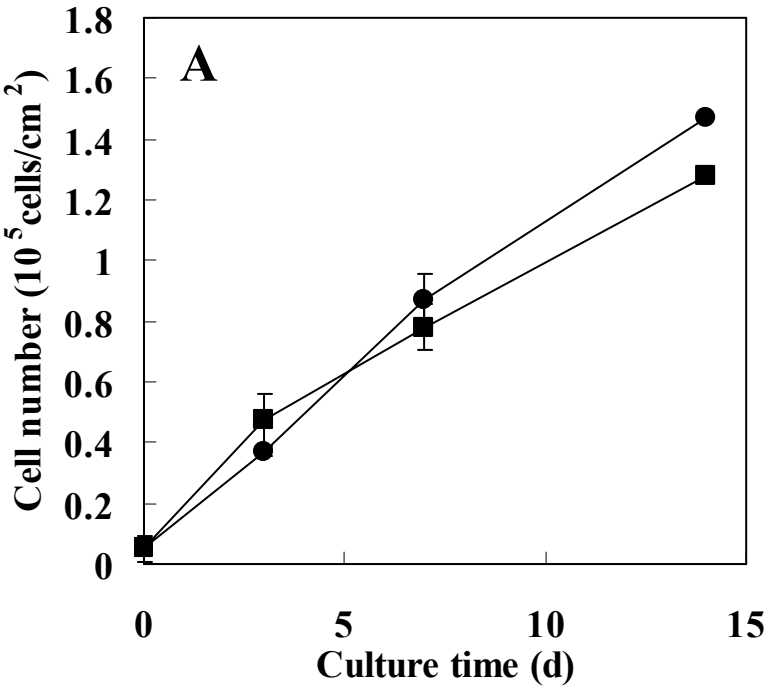




Fig.3


A
Cell number ($10^5$cells/cm$^2$)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
Culture time (d)

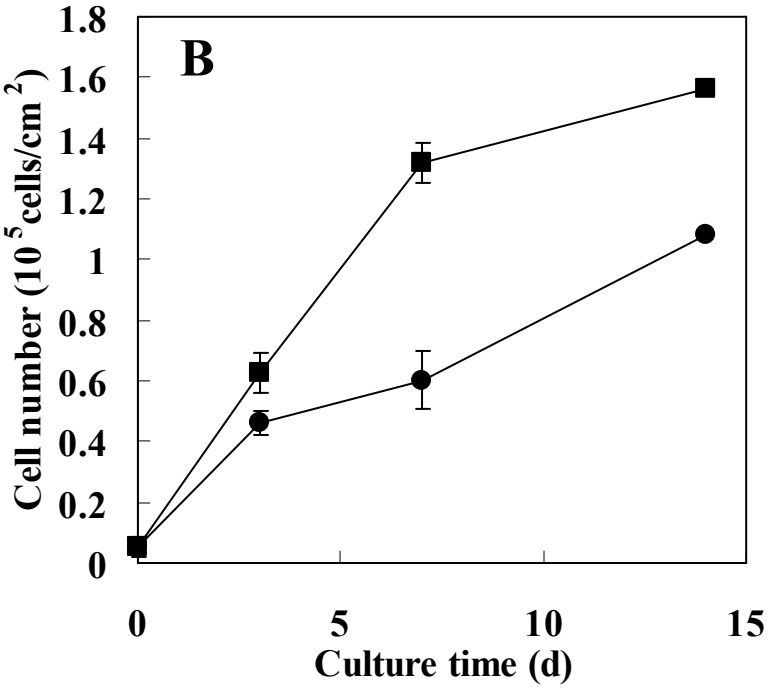
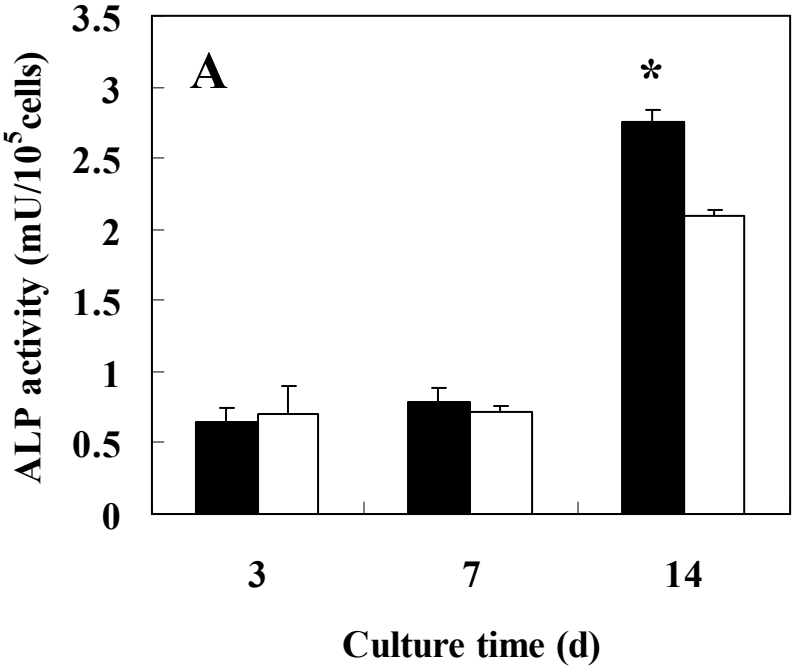

B
Cell number ($10^5$cells/cm$^2$)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
Culture time (d)

Fig.4


A
*
ALP activity (mU/10^5cells)
3.5
3
2.5
2
1.5
1
0.5
0
3
7
14
Culture time (d)

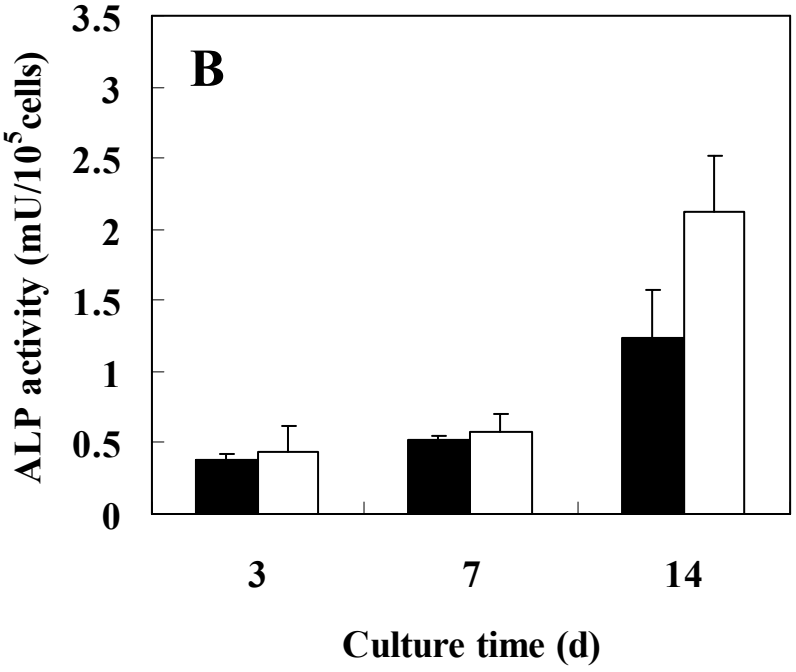

B
ALP activity (mU/10^5cells)
3.5
3
2.5
2
1.5
1
0.5
0
3
7
14
Culture time (d)

Fig.5

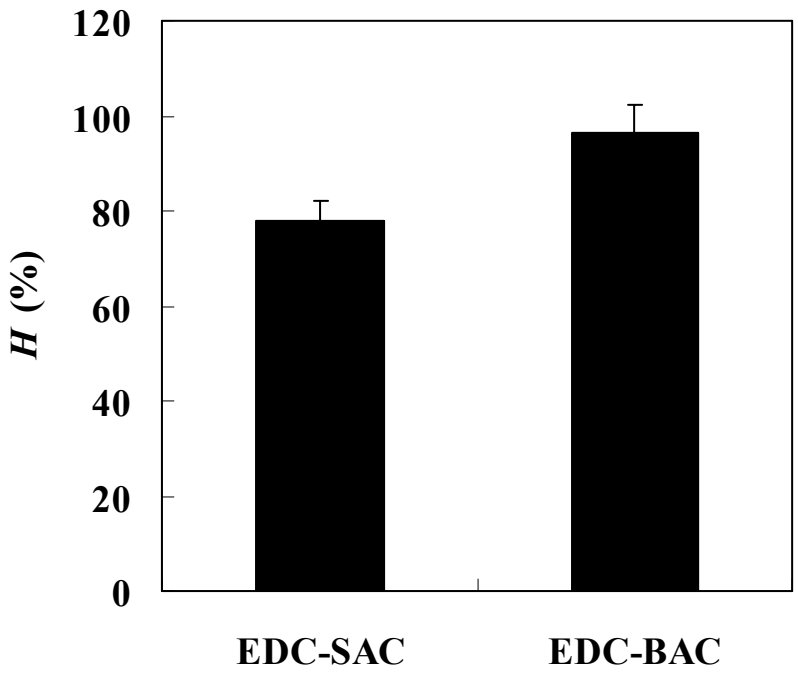



Fig.6

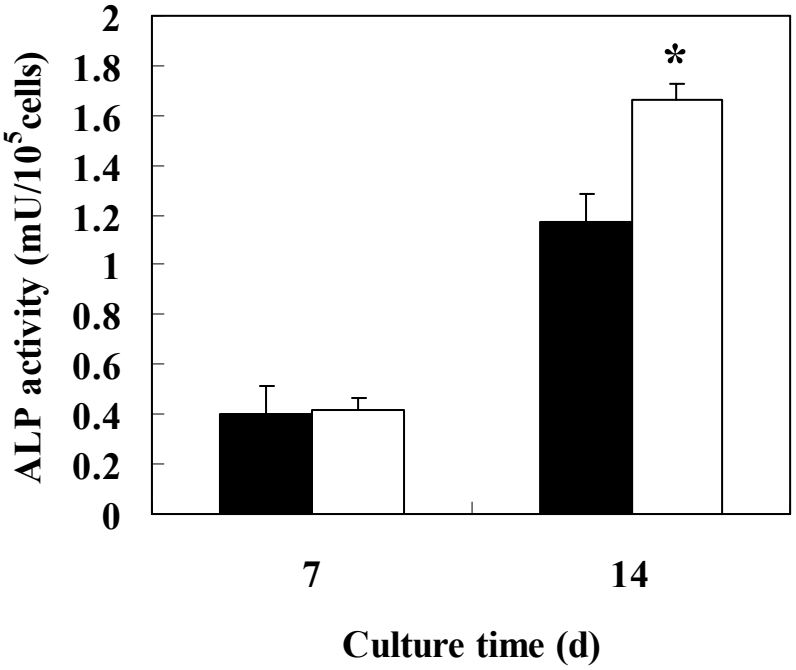

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ALP activity (mU/10^5cells)
*
7
14
Culture time (d)

Fig.7