

Title:

Characteristics of a self-assembled fibrillar gel prepared from red stingray collagen

Running title:

Red stingray collagen fibrillar gel

Authors:

Inwoo BAE ¹, Kiyoshi OSATOMI ², Asami YOSHIDA ¹, Atsuko YAMAGUCHI ², Katsuyasu

TACHIBANA ², Tatsuya ODA ² AND Kenji HARA ^{2*}

Affiliations:

¹Graduate School of Science and Technology, Nagasaki University, 1-14 Bunkyo, Nagasaki 852-8521,

Japan, and ²Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo, Nagasaki 852-8521, Japan

* Corresponding author. Tel: +81-95-819-2828. Fax: +81-95-819-2828.
Email: hara@net.nagasaki-u.ac.jp

ABSTRACT

A translucent collagen gel was formed from a transparent acidic solution of red stingray collagen by adjusting to physiological ionic strength and pH in phosphate buffer, and then incubating at 25–37°C. During fibril formation from red stingray collagen, the turbidity increased when NaCl concentration was increased at constant pH and the rate of fibril formation was accelerated by higher pH or lower NaCl concentration. The T_m of red stingray collagen fibrillar gel was estimated as $44.3 \pm 3.5^\circ\text{C}$, which was higher than that of the collagen solution, 33.2°C . In addition, red stingray collagen gel maintained its shape without melting and was suitable for culture of mouse stromal cells at 37°C .

KEYWORDS: Cell culture, Collagen fibrillar gel, Fibril formation, Fish collagen, Red stingray, Type I collagen.

INTRODUCTION

Collagen is the most abundant protein in animals, accounting for 30% of all proteins in mammals; it occurs in connective tissue, tendons, ligaments, and the cornea as the major protein. A variety of studies have revealed the biochemical properties of collagen molecules and materials in land animals. Type I

collagen, especially, has been used in foods, cosmetics, and medical materials [1, 2]; the main sources of collagens are pig and cow skins and bones.

Recently, marine collagens from the skin and bone of several fish species have been studied [3-6], because marine collagens are thought to guarantee the safety of the source from bovine spongiform encephalopathy (BSE) and because of abundant resources as an alternative collagen source. Most studies have mainly reported the purification and characterization of collagen from marine resources. There are few reports suggesting utilization of fish collagens, owing to the lower denaturation temperature (below 30°C) than mammalian collagen. Use of fish collagen (not gelatin) from the blue shark *Prionace glauca* and from the chum salmon *Oncorhynchus keta*, only has been studied. In these studies the collagen gel was prepared by collagen fibril self-assembly, using fibril-forming ability; *in vitro* the self-assembly of type I collagen molecules takes place when the collagen solution is adjusted to physiological pH, ionic strength, and temperature [7]. It has been suggested that improvement of low thermal stability may result in possible use of collagen gel in biomaterials. The composite collagen gel of shark collagen and pig collagen had better thermal behavior [8]. Yunoki et al. suggested improvement of the thermal stability of gel by adding cross-linking agents during fibril formation and cell culture on the reinforced salmon collagen gel [9]. Meanwhile, we recently found that type I collagen from the skin of red stingray *Dasyatis akajei* has a higher denaturation temperature (T_d , 33.2°C) than that of other fishes [10]. Using this collagen, we have attempted to make a collagen fibrillar gel

which was stable at the physical temperature of human by a fibril-formation process, by adjusting the pH and the NaCl concentration of buffer solution, and the temperature, without use of any additives, to improve the melting temperature. In this study, we investigated the self-assembly of red stingray type I collagen, and then the properties of the collagen gel. On the basis of these results, we also suggest it has potential use as a cell substrate.

Materials and methods

Preparation of type I collagen

Type I collagen was extracted from the skin of red stingray as described in our previous paper [10]. All procedures used to prepare type I collagen were carried out at 4°C. The cleaned skins were extracted with 20 volumes (v/w) of 50 mM acetic acid for three days with magnetic stirring. The extract was centrifuged at 9,000g for 1 h, and the supernatant was salted-out by adding NaCl to a final concentration of 10%. The resulting precipitate was collected by centrifugation at 9,000g for 1 h. The precipitate was dissolved in 20 volumes (v/w) of 50 mM acetic acid and any insoluble material was then removed by centrifugation at 9,000g for 1 h. The supernatant was salted-out by adding NaCl to 20%, and the resulting precipitate was separated by centrifugation as described above. The precipitate was dissolved

in five volumes (v/w) of 50 mM acetic acid. After centrifuging at 9,000g for 1 h, the supernatant was dialyzed against 50 volumes (v/v) of 0.01 M Na₂HPO₄ for three days with a change of solution twice per day. The precipitate was obtained by centrifugation at 9,000g for 1 h, and then lyophilized.

Degree of collagen fibril formation

The degree of collagen fibril formation was examined in at different pH and NaCl concentrations by the method of Yunoki et al. [9] with slight modification. The lyophilized red stingray collagen was dissolved in 50 mM acetic acid to a concentration of 0.8% (w/v) at 4°C, and the collagen concentration was adjusted to 0.2% (w/v) with 70 mM Na-phosphate buffer at different pH (6.1, 6.8, 7.4, 7.9, and 8.5), and NaCl concentrations (0, 35, 70, and 140 mM). To prepare the collagen fibrillar gel, the mixture was poured into a centrifuge tube and incubated at 25°C. After, incubation for one day the amount of collagen fibril was estimated, by BCA assay, from the protein content of the supernatant after centrifuging at 20,000g for 20 min. The degree of collagen fibril formation was defined as the percentage of the decrease of the collagen concentration in the supernatant.

Fibril formation

Fibril formation from red stingray collagen was performed by the method of Yunoki et al. [9] with slight modification. Collagen gel was prepared in micro well plate (diameter 6 mm). Collagen solution (0.8% w/v) was mixed with 70 mM Na-phosphate buffer (pH 6.1, 6.8, 7.4, 7.9, and 8.5) containing NaCl at 0, 35, 70, and 140 mM, using a collagen solution/ Na-phosphate buffer ratio of 1:3 (v/v). The micro well plate was placed in a spectrophotometer (Multiskan Spectrum; Thermo Electron, Tokyo, Japan), and then the resulting fibril formation at 25°C was monitored by the absorbance at 310 nm as the turbidity change.

Preparation of porcine collagen gel

Porcine collagen (0.3% w/v) was purchased from Nitta Gelatin (Cellmatrix Type I-A; Nitta Gelatin, Osaka, Japan). The porcine collagen gel was prepared by the method of Yunoki et al. [9] with slight modification. The porcine collagen solution was mixed with 90 mM Na-phosphate buffer (pH 7.4) containing 210 mM NaCl, using a collagen solution/ Na-phosphate buffer ratio of 2:1 (v/v). The mixture was incubated at 25°C or 37°C for 24 h, and used in the following experiments.

Determination of melting temperature

The thermal stability of the collagen gels was evaluated by determination of the melting temperature (T_m). Calorimetric measurement was performed by differential scanning calorimetry (DSC; DSC 120; Seiko Instruments, Tokyo, Japan). The instrument was calibrated for temperature and enthalpy using indium and tin as standards. To prepare the collagen gel, 0.8% (w/v) collagen solution was mixed 70 mM Na-phosphate buffer (pH 7.4) containing 70 mM NaCl to furnish a final concentration of 0.2% (w/v). The mixtures (80 μ L) were weighed accurately into an aluminium capsule, which was then sealed; an empty capsule was used as reference. The samples were scanned over the range of 15–80°C with a heating rate of 1°C/min. T_m of the collagen gels was estimated from the thermogram. The change of enthalpy (ΔH) was determined from the peak area and expressed in mJ/mg sample material.

Scanning electron microscopy

The collagen fibrils were observed by scanning electron microscopy (SEM; JSM-6380; Jeol, Tokyo, Japan). The collagen fibril was fixed with 2% (v/v) glutaraldehyde in 70 mM phosphate buffer (pH 7.4) for 1 h at room temperature, and then rinsed with distilled water. Dehydration was carried out in a series of ethanol concentrations (60, 70, 80, 90, and 100% v/v), and the ethanol was replaced with isoamyl acetate, followed by drying with liquid CO₂ in a critical-point dryer (HCP-1; Hitachi, Tokyo, Japan). The dried gel was coated with platinum by ion sputtering (JFC-1600; Jeol), and subjected to SEM.

Cell culture

Stromal cells (FLS 5) from mouse fetal liver were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University, Japan. The FLS 5 cells were cultured in RITC 80-7 medium (Research Institute for the Functional Peptide (IFP), Yamagata, Japan) containing 2% fetal bovine serum (FBS; BioSource International, Rockville, MD, USA), 1 µg/ml insulin (human recombinant; MP Biomedical, France), 10 µg/ml transferrin (Calbiochem, CA, USA), and 10 ng/ml epidermal growth factor (EGF; recombinant murine, PeproTech, London, UK) in a humidified atmosphere of 5% CO₂ in air at 37°C. At confluence, FLS 5 cells were treated with 0.1% trypsin and seeded on the collagen gels at a density of 5×10³ cells/well. The cells on the collagen gels were observed by use of an inverted microscope (Axiovert 200, Zeiss, Jena, Germany).

The collagen gels were prepared in 48 well culture plates (Falcon, NJ, USA). Before cell cultivation, collagen gels were preincubated with the same medium for one day at 37°C.

Results

Degree of collagen fibril formation

The effects of pH and NaCl concentration on the degree of collagen fibril formation from red stingray are shown in Fig. 1. The ionic strength was varied by changing the NaCl concentration. Above pH 6.8, the degree of collagen fibril formation ranged from 92 to 96%, and was slightly increased by increasing NaCl concentration at the same pH level. At pH 6.1, in contrast, collagen fibril formation was markedly increased with increasing NaCl concentration.

Fibril formation

During incubation at 25°C, fibril formation was monitored by the absorbance change at 310 nm as the turbidity changed. The effects of pH and NaCl concentration on the rate of fibril formation are shown in Fig. 2. In the pH range 6.1–8.5 at the same NaCl concentration, the rate of fibril formation was increased with increasing pH. A slight decrease of fibril formation rate, however, was observed with increasing the NaCl concentration at constant pH, and it took more time to attain the maximum absorbance in a higher concentration of NaCl. The final absorbance at 310 nm reached high values with increasing concentration of NaCl in the phosphate buffer. In particular, a significant increase in turbidity was observed after 12 min in phosphate buffer at pH 6.1 including 140 mM NaCl, although a change of the turbidity was observed below 70 mM NaCl.

Thermal stability of collagen gel

The DSC curves of red stingray collagen and porcine fibrillar gel are shown in Fig. 3; T_m was calculated from maximum transition point (the endothermic peak) of the thermal melting curves. The T_m of red stingray collagen fibrillar gel was estimated as $44.3 \pm 3.5^\circ\text{C}$, and that of porcine collagen was $51.3 \pm 0.7^\circ\text{C}$.

Observations of collagen fibrillar gel structure by SEM

SEM of red stingray collagen gel is illustrated in Fig. 4. It was apparent that red stingray collagen gel had more slender fibrils, and a more well-formed and a more regular fibril network structure compared with that of porcine collagen.

Cell culture

To examine the effectiveness of the red stingray collagen gel as a cell-culture matrix, FLS 5 cells on red stingray collagen gel were observed for 1, 2, and 4 days, and cell proliferation was compared with

that on porcine collagen gel (Fig. 5). The rate of proliferation of FLS 5 cells on red stingray collagen gel was similar to that on porcine collagen gel. Although significantly different cell shapes were not observed, the cells on red stingray collagen gel were slightly slim and well-stretched in shaped whereas those on porcine collagen gels were more rounded.

Discussion

A translucent collagen gel was formed from a transparent acidic solution of red stingray collagen by adjusting to physiological ionic strength and pH in phosphate buffer, and then incubating at 25–37°C. At pH above 7.4, the degree of fibril formation from red stingray collagen was nearly same and at a high rate (94.5-96.9%) irrespective of NaCl concentration. At pH 6.1, the mixture was maintained in a state of fluidity in phosphate buffer containing 0–70 mM NaCl, but the color of the mixture changed in turbidity and degree of fibril formation was increased to 92% when NaCl concentration reached 140 mM (Fig. 1).

Gross and Kirk [11] showed that neutral phosphate solution of collagen formed a rigid gel when warmed for 30 min at 37°C. In addition, the influence of ionic strength and pH of phosphate buffer was described using the characteristic sigmoid curve obtained from measurement of changes of opacity with time. They demonstrated that optical density was proportional to the amount of collagen

precipitated on formation of collagen fibrils. It was also reported that optical density at 313 nm increased as collagen fibrils were formed, in a typical turbidity curve which is characterized by a lag phase where there is no detectable change in turbidity, a growth phase during which turbidity changes rapidly, and a plateau region where turbidity again remains constant [12]. These studies indicated that the optical density at 313 nm increased as fibrils formed. In our study, fibril formation from collagen under different NaCl concentration and pH conditions was monitored by the absorbance change at 310 nm (Fig. 2). After incubation for 10 min, the collagen gel reached a steady state under all pH and NaCl conditions except pH 6.1, and differences between the absorbances of the steady states were insignificant. In the pH range 6.8-8.5, there was a slight increase in the maximum turbidity with increasing pH and NaCl concentration. These results were inconsistent with those from the degree of fibril formation under different pH and NaCl conditions shown in Fig. 1. However, pH and NaCl concentration of phosphate buffer have little influence on the process of collagen fibril formation. Lag time and the time to attain the maximum turbidity were extended as the concentration of NaCl in the phosphate buffer was increased at constant pH, whereas those times were accelerated by higher phosphate buffer pH at constant NaCl. These results show that the degree of fibril formation in the latent period was not affected by pH and NaCl concentration of nearly neutral phosphate buffer (pH 6.8–8.5 containing 0-140 mM NaCl), but there was a slight difference in the process of collagen fibril formation. These results were similar to those of previous studies using the collagen isolated from rat tail tendon

[12] and calf skin [11]. However, slight differences in the process of collagen fibril formation as a result of changes of pH and NaCl concentration may affect the appearances of collagen fibrils. Williams et al. demonstrated different appearances of collagen fibrils obtained under different conditions of pH and NaCl concentration by electron microscopy [12]. We are also interested in the effects of pH and NaCl concentration on the appearance of red stingray collagen fibril, and have a plan to investigate this further.

In contrast, salmon collagen gave a slightly different result. Fibril formation from salmon collagen increased with pH and the rate of fibril formation was suppressed by high concentrations of NaCl, which were similar to those of our study. However, the maximum turbidity and the degree of fibril formation were reduced by increasing the NaCl concentration [9]. This is explained in the report by Williams et al. - the fibril-formation process is highly sensitive to the source and method of purification of the collagen, to the solvent system employed, and to the conditions under which fibril formation is initiated [12]. Nomura et al. reported that the fibrils and network constructed from shark collagen were different from those formed from pig collagen, and emphasized the need to investigate such differences in terms of differences in molecular structure [13].

As shown in Fig. 3, the T_m of red stingray collagen fibrillar gel was estimated to be approximately $44.3 \pm 3.5^\circ\text{C}$, which was higher than that of the collagen solution, 33.2°C [10]. The fact that the T_m of the collagen fibrillar gel was higher than the denaturation temperature of collagen solution has already been suggested in previous studies [8, 9]. It was suggested that collagen fibrillar gel had

higher heat-stability as a result of the structural stability resulting from assembly of collagen molecules into fibrils. The T_m of red stingray collagen fibrillar gel indicated it was possible to use at 37°C as a cellular matrix. Actually, collagen gel maintained its shape without any melting and was suitable for culture of mouse stromal cells at 37°C. Successful cell culture on fish collagen at 37°C was also reported for salmon fibrillar collagen gel [9, 14]. However, the salmon collagen gel used in cell culture was prepared by adding the EDC, a cross-linking reagent, during fibril formation, because of the low melting temperature of the original salmon collagen gel (23–28°C) [9]. It has also been reported that shark collagen gel could be used as a cell-culture matrix, but cell culture was conducted at 30°C. The shark collagen gel melted at 37°C although its melting temperature was 39–41°C [15]. Therefore, this is the first report to show successful cell culture at 37°C on fish collagen gel to which no reagents were added to improve the thermal stability. In addition, the suitability of red stingray collagen gel as a cell-culture matrix was also confirmed (Fig. 5). FLS 5 cells on red stingray collagen gel showed approximately the same proliferation as on porcine collagen gel and fibroblastic morphology as established in a previous report [16]. The shapes of cells on red stingray collagen gel were, however, slightly differed from those on porcine collagen gel. The cells on the red stingray were somewhat slim and longer, and well-stretched compared with those on porcine collagen gel. This may be because the red stingray collagen fibril network formed from slender collagen fibrils (Fig. 4). Nagai et al. also reported observing different cell shapes on salmon collagen gel. Human periodontal ligament fibroblasts cultured on porcine collagen gel

were slim and spindly, whereas those on the salmon collagen gel were wide and flat. They suggested that the changes of cell morphology could be because of differences in the chemical and mechanical properties of the collagen gel, although fibroblasts have generally a spindle shape on collagen gel [17].

In this study, we prepared collagen gel from red stingray collagen solution by adjusting the pH and NaCl concentration in phosphate buffer and then warming. The gel obtained did not melt at 37°C, and also potential as a cell substrate, suggesting the possibility of use as a collagen-based material as an alternative to porcine collagen gel.

Acknowledgements

The authors thank Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University for providing of mouse stromal cells (FLS 5). This work was supported in part by a Grant-in-Aid for Scientific Research from Nagasaki University, Japan.

REFERENCES

1. Morimura S, Nagata H, Uemura Y, Fahmi A, Shigematsu T, Kida K (2002) Development of an effective process for utilization of collagen from livestock and fish waste. *Process Biochem*

37: 1403–1412

2. Swatschek D, Schatton W, Kellermann J, Muller WEG, Kreuter J (2002) Marine sponge collagen: Isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum. *Eur J Pharm Biopharm* 53: 107–113
3. Muyonga JH, Cole CGB, Duodu KG (2004) Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chem* 85: 81–89
4. Nagai T, Yamashita E, Taniguchi K, Konamori N, Suzuki N (2001) Isolation and characterization of collagen from the outer skin waste material cuttlefish (*Sepia Lycidas*). *Food Chem* 72: 425–429
5. Nagai T, Araki Y, Suzuki N (2002) Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chem* 78: 173–177
6. Senaratne LS, Park PJ, Kim SK (2006) Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresour Technol* 97: 191–197
7. Kadler KE, Holmes DF, Trotter JA, Chapman JA (1996) Collagen fibril formation. *Biochem J* 316: 1–11
8. Nomura Y, Toki S, Ishii Y, Shirai K (2000) Improvement of the material property of shark type I collagen by composing with pig type I collagen. *J Agric Food Chem* 48: 6332–6336
9. Yunoki S, Nagai N, Suzuki T, Munekata M (2004) Novel biomaterial from reinforced salmon

- collagen gel prepared by fibril formation and cross-linking. *J Biosci Bioeng* 98: 40–47
10. Bae I, Osatomi K, Yoshida A, Osako K, Yamaguchi A, Hara K (2008) Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. *Food Chem* 108: 49–54
 11. Gross J, Kirk D (1958) The heat precipitation of collagen from neutral salt solutions: some rate regulating factors. *J Biol Chem* 233: 355–360
 12. Williams BR, Gelman RA, Poppke DC, Piez KA (1978) Collagen fibril formation. *J Biol Chem* 253: 6578–6585
 13. Nomura Y, Toki S, Ishii Y, Shirai K (2002) The physicochemical property of shark type I collagen gel and membrane. *J Agric Food Chem* 48: 2028–2032
 14. Nagai N, Yunoki S, Satoh Y, Tajima K, Munekata M (2004) A method of cell- sheet preparation using collagenase digestion of salmon atelocollagen fibrillar gel. *J Biosci Bioeng* 98: 493–496
 15. Nomura Y, Kitazume N (2002) Use of shark collagen for cell culture and zymography. *Biosci Biotechnol Biochem* 66: 2673–2676
 16. Ohneda O, Yanai N, Obinata M (1990) Microenvironment created by stromal cells is essential for a rapid expansion of erythroid cells in mouse fetal liver. *Development* 110: 379–384

17. Nagai N, Mori K, Satoh Y, Takahashi N, Yunoki S, Tajima K, Munekata M (2007) In vitro growth and differentiated activities of human periodontal ligament fibroblasts cultured on salmon collagen gel. *J Biomed Mater Res Part A* 82: 395–402

FIGURE CAPTIONS

Fig. 1 Effect of pH and NaCl concentration on degree of fibril formation by red stingray fibrillar collagen gel. The concentrations of NaCl in Na-phosphate buffer were 0 mM (*dotted box*), 35 mM (*fulled box*), 70 mM (*open box*) and 140 mM (*striped box*), respectively. The degree of collagen fibril formation was determined as the percentage of the decrease of the collagen concentration in the supernatant as a result of fibril formation. Each sample was run in triplicate

Fig. 2 Effect of Na-phosphate buffer pH (*diamonds 6.1, squares 6.8, circles 7.4, triangles 7.9, crosses 8.5*) and NaCl concentration on the progress of red stingray type I collagen fibril formation

Fig. 3 Differential scanning calorimetry thermograms: (A) red stingray collagen gel; (B) porcine collagen gel. The change of enthalpy (ΔH) was determined from the peak area and expressed in mJ/mg sample material. The red stingray collagen solution (0.8% w/v) was mixed with 70 mM Na-phosphate buffer (pH 7.4) containing 70 mM NaCl, using a collagen solution/ Na-phosphate buffer ratio of 1:3 (v/v). The porcine collagen solution was mixed with 90 mM Na-phosphate buffer (pH 7.4) containing 210 mM NaCl, using a collagen solution/ Na-phosphate buffer ratio of 2:1 (v/v)

Fig. 4 Scanning electron micrographs of collagen fibrils: (A) red stingray collagen gel; (B) porcine collagen gel. The red stingray collagen solution (0.8% w/v) was mixed with 70 mM Na-phosphate buffer (pH 7.4) containing 70 mM NaCl, using a collagen solution/ Na-phosphate buffer ratio of 1:3 (v/v). The porcine collagen solution was mixed with 90 mM Na-phosphate buffer (pH 7.4) containing 210 mM NaCl, using a collagen solution/ Na-phosphate buffer ratio of 2:1 (v/v). The mixtures were incubated at 25°C for 24 h. Magnification, 15,000x; *bars*, 1 μ m

Fig. 5 Cell proliferation on collagen gel matrix for 1, 2, and 4 days: (A) red stingray collagen gel; (B) porcine collagen gel. The mouse stromal cells (FLS 5) were cultured on the gels at 37°C. The gels were prepared as described in the caption to Fig. 4

Fig.1

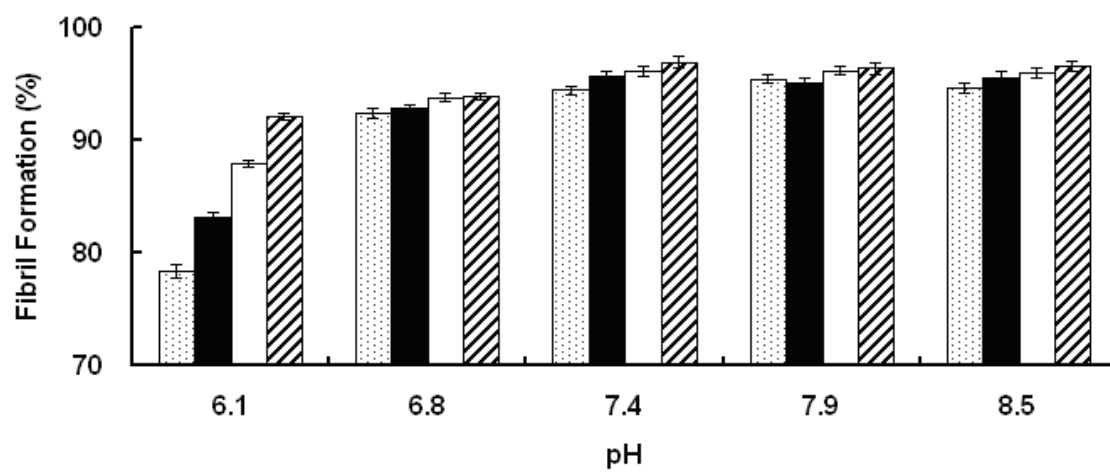


Fig.2

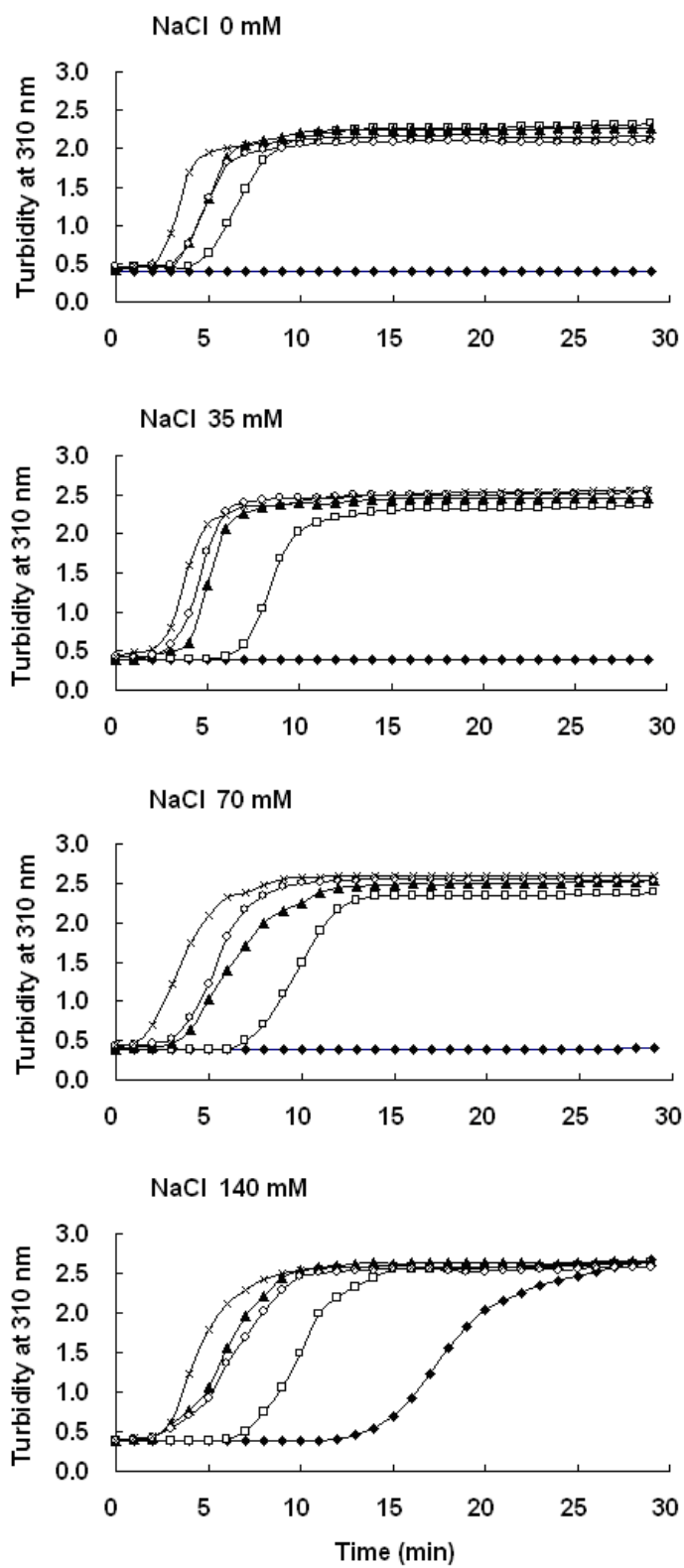


Fig.3

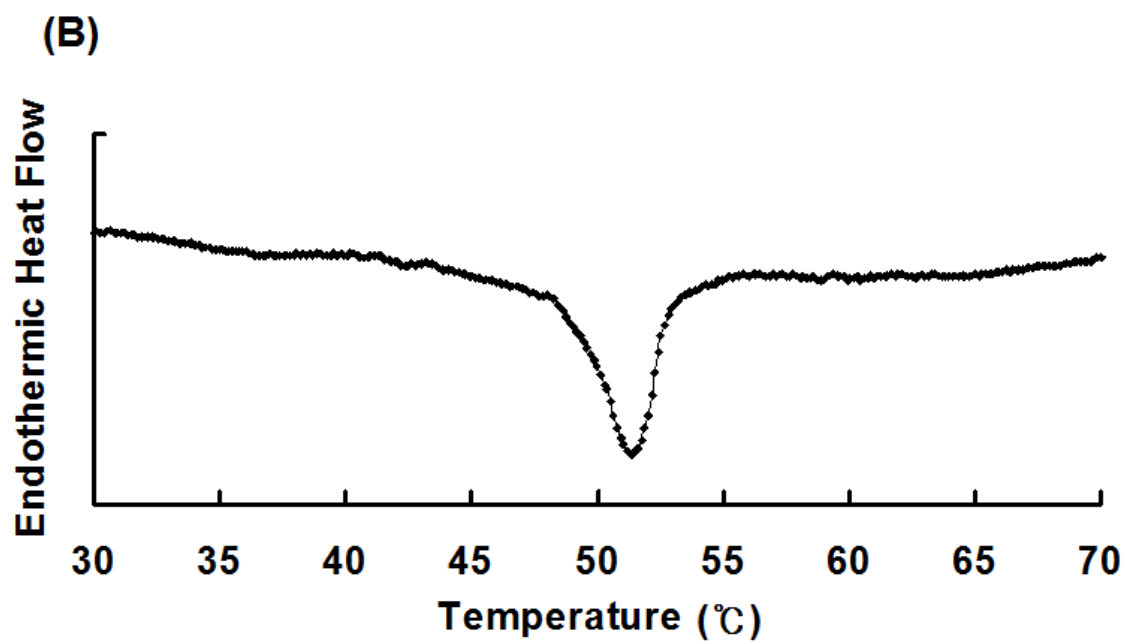
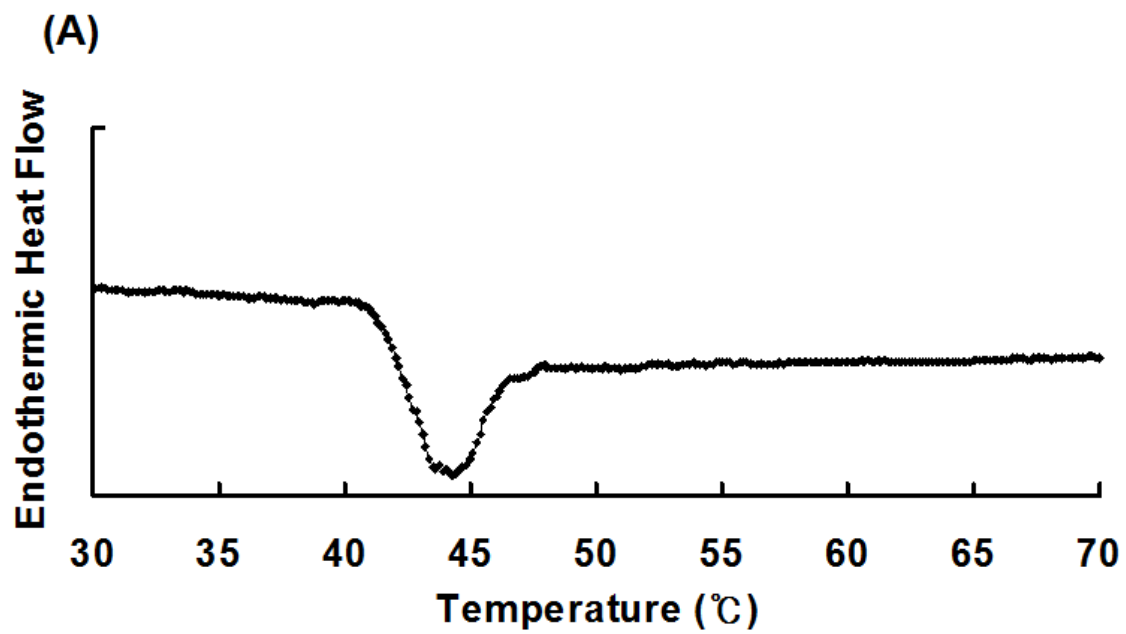
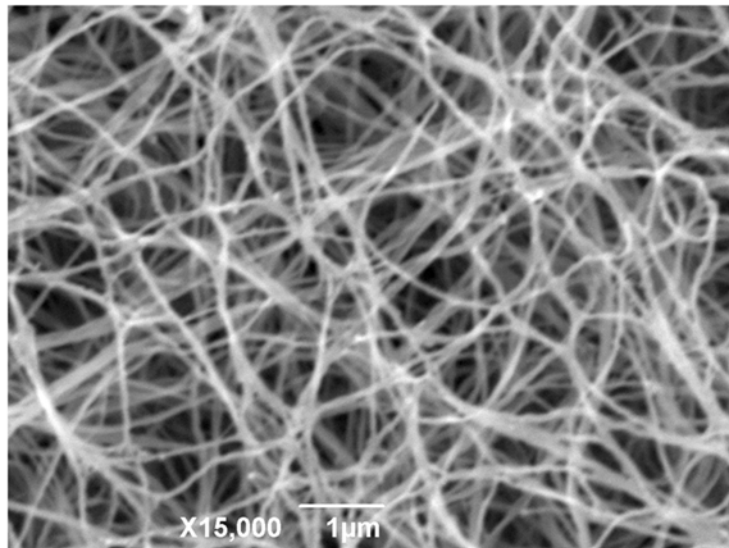


Fig.4

(A)



(B)

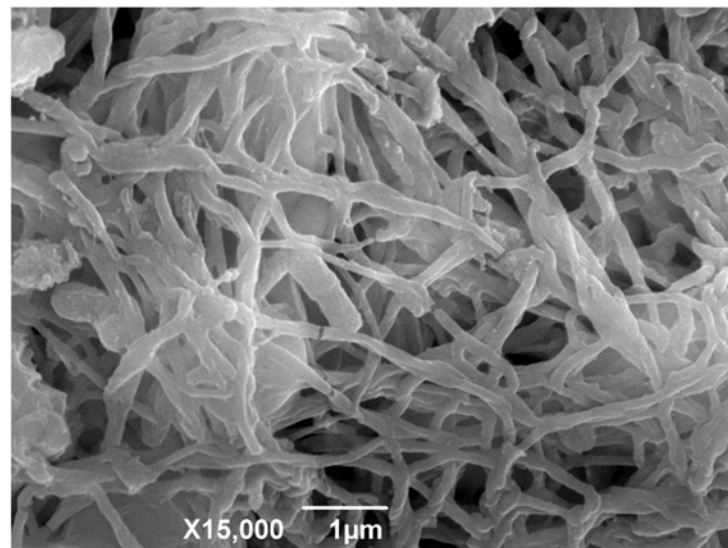


Fig.5

