Regular article

Swim bladder collagen forms hydrogel with macroscopic superstructure by diffusion induced fast gelation

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ABSTRACT: Marine collagen has been attracting attention as medical materials in recent times due to the low risk of pathogen infection compared to animal collagen. Type I collagen extracted from swim bladder of Bester sturgeon fish has excellent characteristics such as high denaturation temperature, high solubility, low viscosity and extremely fast rate to form large bundle of fiber at certain conditions. These specific characteristics of swim bladder collagen (SBC) permit us to create stable, disk shaped hydrogels with concentric orientation of collagen fiber by the controlled diffusion of neutral buffer through collagen solution at room temperature. However, traditionally used animal collagens, e.g. calf skin collagen (CSC) and porcine skin collagen (PSC) could not form any stable and oriented structure by this method. The mechanism of superstructure formation of SBC by diffusion induced gelation process has been explored. The fast fibrillogenesis rate of SBC causes a quick squeezing out of solvent from the gel phase to the sol phase during gelation, which builds an internal stress at the gel-sol interface. The tensile stress induces the collagen molecules of gel phase to align along the gel-sol interface direction to give this concentric ring-shaped orientation pattern. On the other hand, the slow fibrillogenesis rate of animal collagens due to the high viscosity of the solution does not favor the ordered structure formation. The denaturation temperature of SBC increases significantly from 31^{o}C to 43^{o}C after gelation, whereas that of CSC and PSC was found to increase a little. Rheology experiment shows that SBC gel has storage modulus larger than 15 kPa. The SBC hydrogels with thermal and mechanical stability have potentials as bio-materials for tissue engineering applications.

Key words: Hydrogel, swim bladder collagen, superstructure, diffusion, denaturation temperature.

1. INTRODUCTION

Hydrogels, a class of soft and wet material, are considered to be the most promising smart bio-materials due to their similarity to soft bio-tissues. Developments of hydrogels in last decade[1-5] greatly enhanced the applications of this material in various fields, including artificial organs, drug delivery, regenerative medicine etc.[6-9] Collagen-based hydrogels for biomedical application are becoming very hot topic in medical research because of their low antigenic activity, high cell adhesion properties, biocompatibility, and biodegradability.[10,11] Numerous specific functions of many bio-tissues are strongly dependent on the anisotropic superstructure of fiber-forming collagen.[12,13] The most common sources of collagen for biomaterials and tissue engineering are bovine skin and tendons, porcine skin, and rat tail.[11] In recent times, the use of collagen and collagen-derived products from land-based animal calls into question because of the emergence of zoonosis such as bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD), avian influenza diseases etc.[14,15] Religious beliefs also restrict the usage of porcine or bovine collagens. Marine resource has been attracting attention at the very recent times as a smart alternative of animal collagen due to their low risk of pathogen infection and no religious obstruction.[16]

A huge source of marine-based collagen is now from the wastes of seafood industry. Recently, Zhang *et al.*[17] have found that the swim bladder of Bester sturgeon fish (a hybrid sturgeon of *Husohuso* x *Acipenserruthenus*) is a large source of type I collagen (18.1% on a wet weight basis, 37.7% on a dry weight basis), which has relatively high thermal stability (32.9^{o}C by CD spectroscopy). In addition, apart from the conventional animal collagen, this swim bladder collagen (SBC) has excellent characteristics such as high solubility and homogeneity, low viscosity and extremely fast rate to form large bundle of fiber at certain conditions. However, collagens from other tissues (scales, skin, muscle, digestive tract,

notochord and snout cartilage) of Bester sturgeon do not show those interesting properties.[17] Until now, hydrogels from marine-based collagen have hardly been developed because of the poor availability in comparison with the land-based animal collagen that is abundant in the market.[18,19] Being a marine sourced atelocollagen, SBC is expected to have low antigenicity with less risk of pathogen infection and thus, would be suitable for medical application.[16] However, the immunostimulant characteristics may not always depend only on the terminal group, the removal of telopeptide group would be preferable to make a biomaterial with relatively better safety profile.[20,21] In this work, we focus on developing collagen hydrogels with ordered structure by utilizing this marine sourced type I atelocollagen that has the distinguished properties.

Many efforts have been made for creating ordered structure in collagen-based materials by various methods, including dialysis, shear, hydrodynamic flow, electric field, electro-spinning and magnetic field techniques.[22-29] In this work, we use the diffusion-induced gelation to prepare SBC hydrogels. It is well-known that negatively charged polyelectrolytes having rigid nature, such as DNA, alginate, and poly(2,2′-disulfonyl-4,4′-benzidine terephthalamide) (PBDT) form physical hydrogels when Ca^{2+} ions are allowed to diffuse into these polymer solutions.[30-37] Furthermore, these rigid molecules after gelation are orientated perpendicular to the diffusion direction of Ca^{2+} ion.[34-37] This specific superstructure formation during diffusion-induced gelation has been related to the syneresis effect of gelation.[37] That is, the complexation of negatively charged rigid macromolecules with Ca^{2+} leads to gelation, which induces shrinkage of the gel phase. Since the sol phase does not shrink, an internal stress is built at the sol-gel interface, where the sol phase exerts a tension to the gel phase and the gel phase exerts a compression to the sol phase. As a result, the macromolecules in the gel phase orient along the tensile direction. Our previous study also revealed that, when we

develop programmed swelling mismatch in different regions of a hydrogel containing semi-rigid macromolecules, the mismatch induced internal stress determine the orientation of macromolecules in respective regions.[5]

Although different rigid macromolecules are successfully oriented by diffusion induced gelation process, but still now, it remains a challenge to make oriented structure in collagen hydrogel due to some limitations of animal collagen. In this work, we intend to develop collagen hydrogels with ordered structure based on this mechanism by utilizing the distinguished properties of SBC. Previous study has clarified that SBC molecules are in stable triple-helix molecular form in acidic solution, and they self-assemble into fibrils in neutral buffer.[17] This is because in acidic solution the collagen is positively charged and in the neutral buffer, it becomes almost neutral, which favors fibril formation.[38,39] Thus, if we perform controlled diffusion of neutral buffer into acidic SBC solution, we expect super structure formation of SBC molecule by quick fibrillogenesis of the rigid SBC molecule.

2. EXPERIMENTAL SECTION

Materials: Type I collagen (atelocollagen) was extracted from the swim bladder of Bester sturgeon fish according to the previously reported protocol.[17] The swim bladder collagen was denoted as SBC. Type I calf skin collagen, CSC (tropocollagen, Sigma Aldrich, Japan) and Type I porcine skin collagen, PSC (atelocollagen, Nippi Co. Ltd.) were used as received without further purifications. Analytical grade Na_2HPO_4 and NaH_2PO_4 (Wako Pure Chemical Industries Ltd., Japan**)** were used as received for the preparations of Na-phosphate buffer solution of pH 7.2. Concentrated HCl **(**Wako Pure Chemical Industries Ltd., Japan**)** was used to prepare aqueous HCl solution of pH 2.5 for the preparation of collagen solutions. All the aqueous solutions were prepared using ultrapure deionized water.

Diffusion induced gelation process: To prepare collagen solution, a prescribed amount of collagen was dissolved in *aq.* HCl (pH 2.5). The mixture was left for 3 days without any external perturbations at room temperature (25^{o}C) to get a homogenous solution. After that, a drop (~20 µL) of collagen solution was placed on a glass plate and covered by another glass plate with a gap distance of 0.5 mm, which was controlled by two silicone spacer, as shown in Figure 1. The disk-shaped collagen solution was in contact with the glass plates with an initial diameter about ~6 mm. Gelation of collagen was performed by introducing 0.1 M Na-phosphate buffer (pH 7.2) into the reaction cell from the peripheral part of collagen solution. Gelation progressed from periphery to center of the collagen solution by the diffusion of buffer. After 2 hours, disk shaped collagen hydrogel with diameter ~6 mm and thickness 0.5 mm was formed. For rheological study, we have prepared collagen gel of thickness 1 mm. All the SBC gels were prepared at room temperature (25^{o}C). However; CSC and PSC gels were prepared at 25^{o}C and 34^{o}C. To prepare CSC and PSC gels at 34^{o}C, the collagen and buffer solutions were pre-incubated at that temperature for 10 minutes before starting gelation.

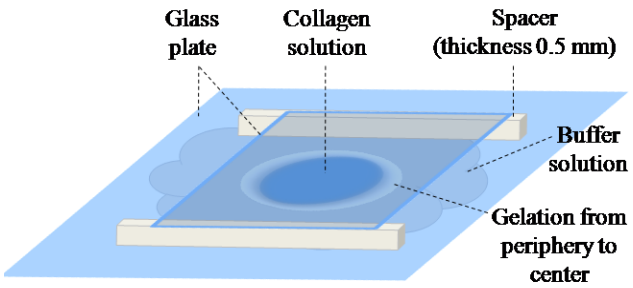

Figure 1 Set up for the diffusion induced gelation process of collagen solution. The diameter of the disc-shaped collagen solution was about 6 mm.

Characterization:

Structure

Time dependent structural change during gelation was monitored under the polarizing optical microscope, POM (Nikon, LV100POL). A color sensitized 530 nm tint plate was used to distinguish collagen orientation. The birefringence at different regions of hydrogel was

148 measured quantitatively from the retardation values using a Berek compensator in POM.
149 Scanning electron microscopy (SEM) (JSM-6010LA, JEOL Ltd.) was applied to study the
150 morphology and orientation of collagen fibril. To prepare the sample for SEM observation,
151 the samples were fixed by 0.1 % (v/v) *aq.* glutaraldehyde for 24 hours and freeze-dried using
152 a freeze drying device (Advantage XL-70, VirTis freeze-dryer) and finally, coated with gold
153 using an ion-sputtering device (E-1010, Hitachi, Japan). The shape of the sample did not
154 change after freeze drying.

155 **Fibrillogenesis**

156 Fibrillogenesis rate of collagen was studied by monitoring the turbidity change of an equal
157 volume mixture of 0.3 wt % collagen and 0.1 M Na-phosphate buffer (pH 7.2) at 320 nm
158 using a quartz cell of 1 cm path length in UV spectrophotometer (UV-1800, Shimadzu UV
159 Spectrophotometer).

160 **Solution properties**

161 Transparency of collagen solution was determined from the turbidity measurement at 320
162 nm. The dynamic viscosity of collagen solution was measured at 25^{o}C using rheometer
163 (ARES-100FRT) by strain controlled steady rate sweep test with a cone and plate geometry
164 of 25 mm diameter, 0.054 mm gap distance and 0.04 radians cone angle, covering a shear rate
165 ranges from 0.1 s^{-1} to 10 s^{-1}.

166 **Thermal stability**

167 The denaturation temperature of collagen solution and collagen hydrogel was determined by
168 differential scanning calorimetry, DSC (SII X-DSC7000, SII Nanotechnology Inc.).

169 **Mechanical property**

170 To study the mechanical strength of SBC hydrogel, a dynamic frequency sweep test was
171 performed from 0.25 s^{-1} to 100 s^{-1} with a shear strain of 0.2 % in the parallel plate geometry

at 25°C. A disk shaped SBC gel of thickness 1 mm and diameter 6 mm was adhered to the plates using glue for measurement.

3. RESULTS AND DISCUSSION

When 0.1 M Na-phosphate buffer solution (pH 7.2) was introduced into the periphery of 4 wt% SBC solution, gelation started immediately from the peripheral part of collagen solution, which could be confirmed from the turbidity appearance as shown in Figure 2(I). Gelation continued to progress from the outside to the inside as the buffer solution slowly diffused into the collagen solution.

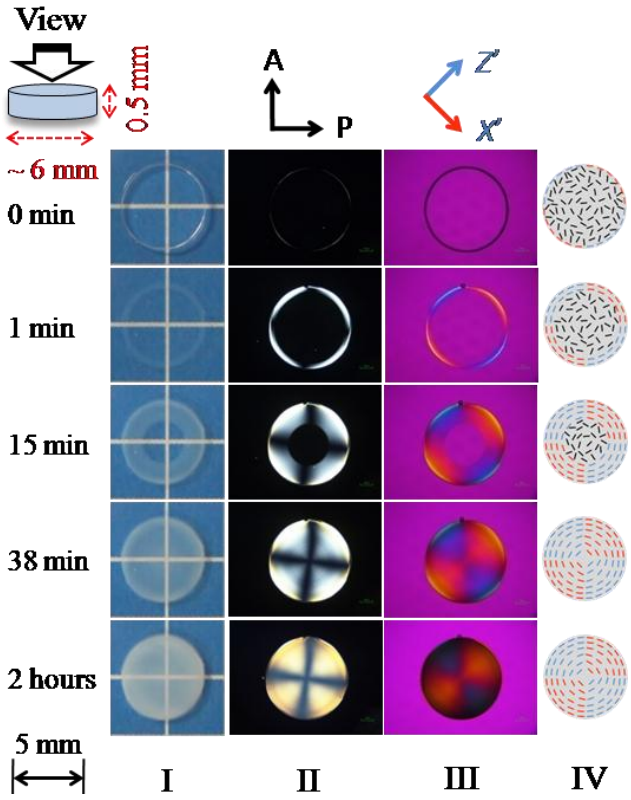

Figure 2 (I) Photographic images of 4 wt% SBC hydrogel at different gelation time. (II, III) POM images under crossed polarizer in absence (II) and presence (III) of color sensitized 530 nm tint plate. All the images are in same scale as shown in the bottom left part. (IV) Illustrations of orientation structure of SBC identified by POM. A: analyzer, P: polarizer. X' and Z': fast and slow axes of tint plate, respectively.

To confirm the presence of oriented structure, we observed the time lapse of gelation processes under POM using crossed polarizer (Figure 2(II)). Before the addition of buffer, no birefringence was observed except at the periphery of collagen solution, indicating that the 4 wt% SBC solution is isotropic, well below the liquid crystalline (LC) concentration. This is in consistent with the result by M.M. Giraud-Guille *et al*.,[40-41] who found that the LC phase of the type I rat tail collagen appears at critical concentration of 8~8.5 wt% in 0.5 M acetic acid (pH 2.5). The peripheral circular birefringence is considered as the edge effect of concentrated polymer solution. The collagen molecules of rigid triple helix assembled at the edge of liquid-air interface to show this ring shaped thin birefringence.

The cross patterned of strong birefringence were observed as the gelation progressed from the periphery to center (Figure 2(II)), which indicates the formation of radially or concentrically oriented structure during the diffusion induced gelation process. To confirm the orientation direction, we used color sensitized 530 nm tint plate during POM observation. Denoting that collagen has a positive birefringence[42], the alternative ring shaped blue (quadrants 2 and 4) and orange (quadrants 1 and 3) colors in POM images (Figure 2(III)) indicate that the collagen molecules orient along and perpendicular to the tint polymer direction, respectively. That is, the collagen is oriented concentrically in the birefringence ring (Figure 2(IV)), in similar to previously reported results on other rigid molecules.[37] When the gelation reached the center of the sample at 38 minutes, the concentric orientation was developed in the whole sample. Rigid triple helix collagen molecules have positive charges at acidic condition.[38] Once the collagen molecules meet with the neutral buffer at the diffusion front, they form aggregated fibers by neutralization. The *syneresis* effect during fiber formation process is considered to be responsible for this super structure formation, in similar to previously reported mechanism.[5] The fibrillogenesis process causes solvent transportation

212 microscopically, and thus a swelling mismatch is built at the sol/gel interface which insists
213 ordered structure formation. It should be mentioned that the volume change of the gel by
214 contraction during gelation is difficult to observe at macroscopic scale. This is probably due
215 to relatively strong adhesion of the collagen gel to the glass wall of the reaction chamber,
216 which prevents the overall volume change of the gel.
217
218 The *syneresis* induced swelling mismatch can be justified when we observe the gelation front
219 line *in situ* very carefully. A thin line of orange color (indicated by white arrow) ahead of the
220 blue color can be identified at the diffusion front of sol-gel interface (Figure 3(a)(i)). This
221 indicates the presence of radial orientation of collagen ahead of the gelation front as
222 illustrated in Figure 3(a)(ii), which gradually converted into concentric orientation and
223 stabilized with time by forming fiber. During fibrillogenesis process, the contracting gel
224 phase experiences a tensile stress from the very adjacent sol phase. Oppositely, sol phase
225 experiences a compressive stress from the contracting gel phase. Those opposite forces create
226 the concentric and radial orientation in the gel phase and sol-gel boundary, respectively
227 (Figure 3(a)(ii)).
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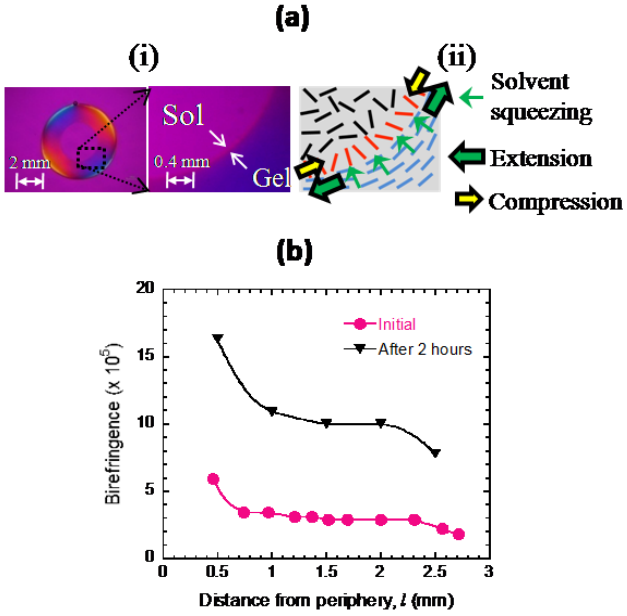


Figure 3 (a) POM image (i) of the diffusion/gelation front region under magnified lens and the corresponding schematic representation (ii) of orientation structure of SBC identified by POM. **(b)** The birefringence of 4 wt% SBC gel at the position right behind the advancing gelation front which corresponds to the initially formed structure (denoted as initial in the Figure) and after 2 hours gelation vs. distance from periphery to center, *l*.

The birefringence increases with time. The initial birefringence right behind the gelation front, which means the structure newly formed with the progress of the diffusion front, is shown in Figure 3(b). The birefringence obtained after 2 hours gelation is about ~3 times higher than the initial birefringence. The turbidity of the gel also increases drastically during this process (Figure 2(I)). This observation suggests that fibrillogenesis process continues for the longer period of time until they reach their characteristic fiber size.

To understand the effect of collagen concentration on superstructure formation, we have prepared hydrogel from low to high concentrations of SBC (1.2 ~ 4 wt%). At 1.2 wt% SBC, the hydrogel only showed weak and irregular birefringence (Figure 4(a)). From 1.5 wt%,

distinct cross-patterns were observed, indicating the formation of concentric structure. Therefore, the minimum concentration of SBC required to form a well oriented gel is around 1.5 wt%. In addition, at 1.5 wt%~3 wt%, but not at 4 wt%, a thin radially oriented layer can be noticed at the peripheral region (at the boundary of the gel and the buffer). As the viscosity of 1.5 ~ 3 wt% is lower than 4 wt%, this may make it possible for a little outflow of collagen solution at the periphery by the strong diffusion of buffer, which creates this radial orientation at the outer part of hydrogel.

It could be observed from Figure 4(a) that the birefringence brightness changes with the concentrations of SBC. The brightness of periphery of hydrogel is stronger than that of the center. The birefringence variation with the distance from periphery to center (*l*) of SBC hydrogels of various concentrations is shown in Figure 4(b). For all the concentrations of SBC, the birefringence decreases with *l*, which indicates that a gradient of orientation degree is created by the diffusion induced gelation process. Up to ~1 mm distance, birefringence decreases sharply. This is because that the diffusion velocity of buffer sharply decreases at the beginning as the gel width increases and therefore, the rate of fibrillogenesis also decreases. So the swelling mismatch created from *syneresis effect* also decreases which generates low internal tensile stress in the gel phase. Hence the orientation degree decreases. However, at the end (close to the center of the gel), birefringence decreases sharply. We considered that at the central part of the gel, the collagen molecules are subjected to tensile stress from all directions, which leads to the formation of poorly oriented structure.

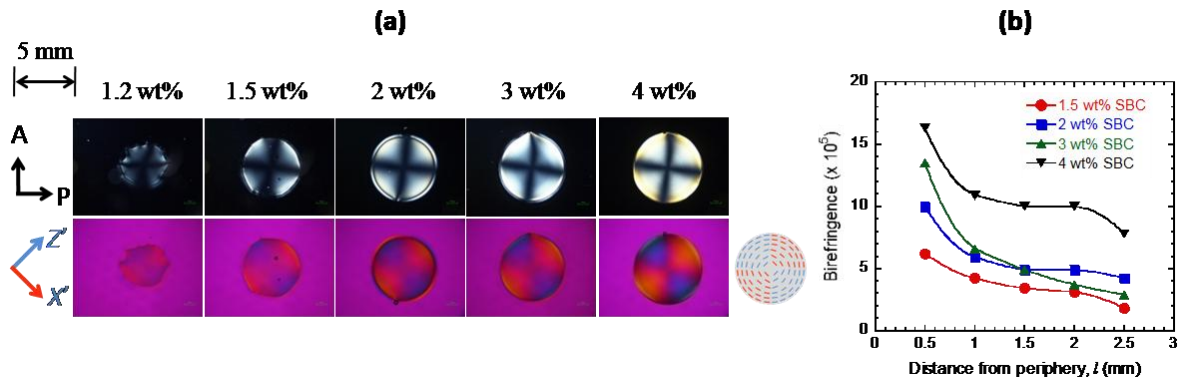


Figure 4 (a) POM images (crossed polarizer both in absence and presence of tint plate) of SBC hydrogels (1 hour gelation) prepared at 25^{o}C having concentration ranges from 1.2 wt% to 4 wt%. Illustration of orientation structure of SBC identified by POM is shown on the right side of POM images. A: analyzer, P: polarizer, X' and Z': fast and slow axes of the tint plate, respectively. All the POM images are in same scale as shown in the top left part. **(b)** The birefringence variation of SBC hydrogel (2 hours gelation), with the distance from periphery to center (*l*) of the hydrogel.

Diffusion induced superstructure formation in SBC hydrogel is quite similar to our previous study,[37] where the binding of negatively charged PBDT molecule with Ca^{2+} ion creates ring shaped concentric orientation pattern. According to our knowledge, it is the first success in creating concentric ring pattern macroscopic superstructure in collagen hydrogel by diffusion induced gelation. Attempt by Furusawa *et al.*[22] demonstrated that, diffusion of buffer through the type I bovine dermis collagen solution made phase separated tubular pores aligned parallel to the growth direction of the gel. This result suggests that different sources of collagen have big impact for the creation of oriented structure. So we further performed diffusion induced gelation in some traditionally used animal collagens as control experiment. For this purpose, we used calf skin collagen (CSC) in tropocollagen form and porcine skin collagen (PSC) in atelocollagen form. We found that both CSC and PSC only form very weak,

non-self-standing gels. Figure 5 shows the POM images of 2 wt% CSC and PSC samples prepared at 25°C and 34°C. The samples prepared at 34°C are more turbid than that prepared at 25°C (Figure 5), which indicates that these collagens have better fibrillogenesis capacity at higher temperature. Interestingly, no birefringence is observed in all cases, except a thin weak concentric birefringence at the periphery of PSC gel prepared at 34°C, indicating that almost random structure is formed in both CSC and PSC hydrogel.

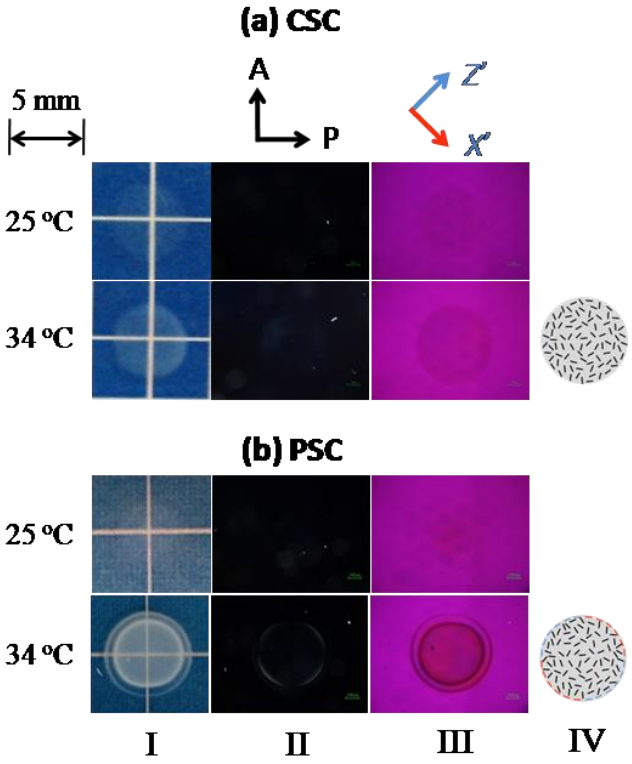


Figure 5 The photographs (I) and POM images (II, III) of **(a)** 2 wt% calf skin collagen (CSC) hydrogel and **(b)** 2 wt% porcine skin collagen (PSC) hydrogel prepared by 3 hours gelation at 25°C and 34°C. Illustrations (IV) of orientation patterns of collagen identified by POM are shown on the right side of POM images. A: analyzer, P: polarizer, X' and Z': fast and slow axes of the tint plate, respectively. All the images are in same scale as shown in the top left part.

We have also confirmed orientation and morphology of fibril structure through SEM observation. Figure 6 (a) and (b) show the SEM images of 4 wt% SBC solution and hydrogel, respectively. All the samples were fixed by glutaraldehyde before performing SEM. SBC solution does not contain any fibril, rather it makes cluster of network polymer (Figure 6(a)), may be formed by glutaraldehyde crosslinking process. SBC hydrogel made by reaction-diffusion (RD) process contains solely of collagen fibrils, which are beautifully aligned perpendicular to the diffusion direction with an almost homogeneous fibril diameter of ~200 nm (Figure 6(b)). On the other hand, both CSC (Figure 6(c)) and PSC (Figure 6(d)) gels contain randomly oriented collagen fibril with inhomogeneous distribution of fibril size.

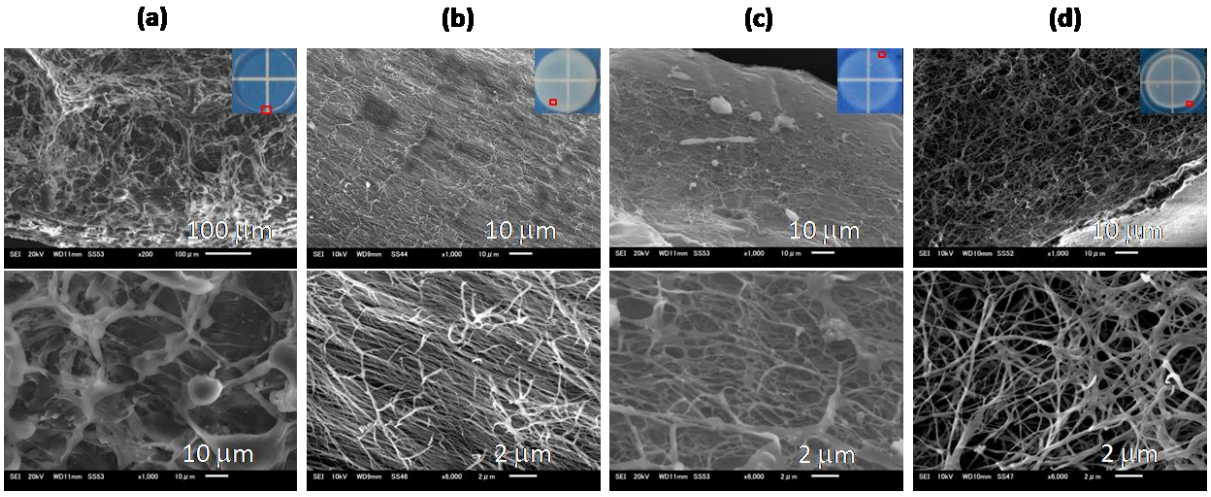
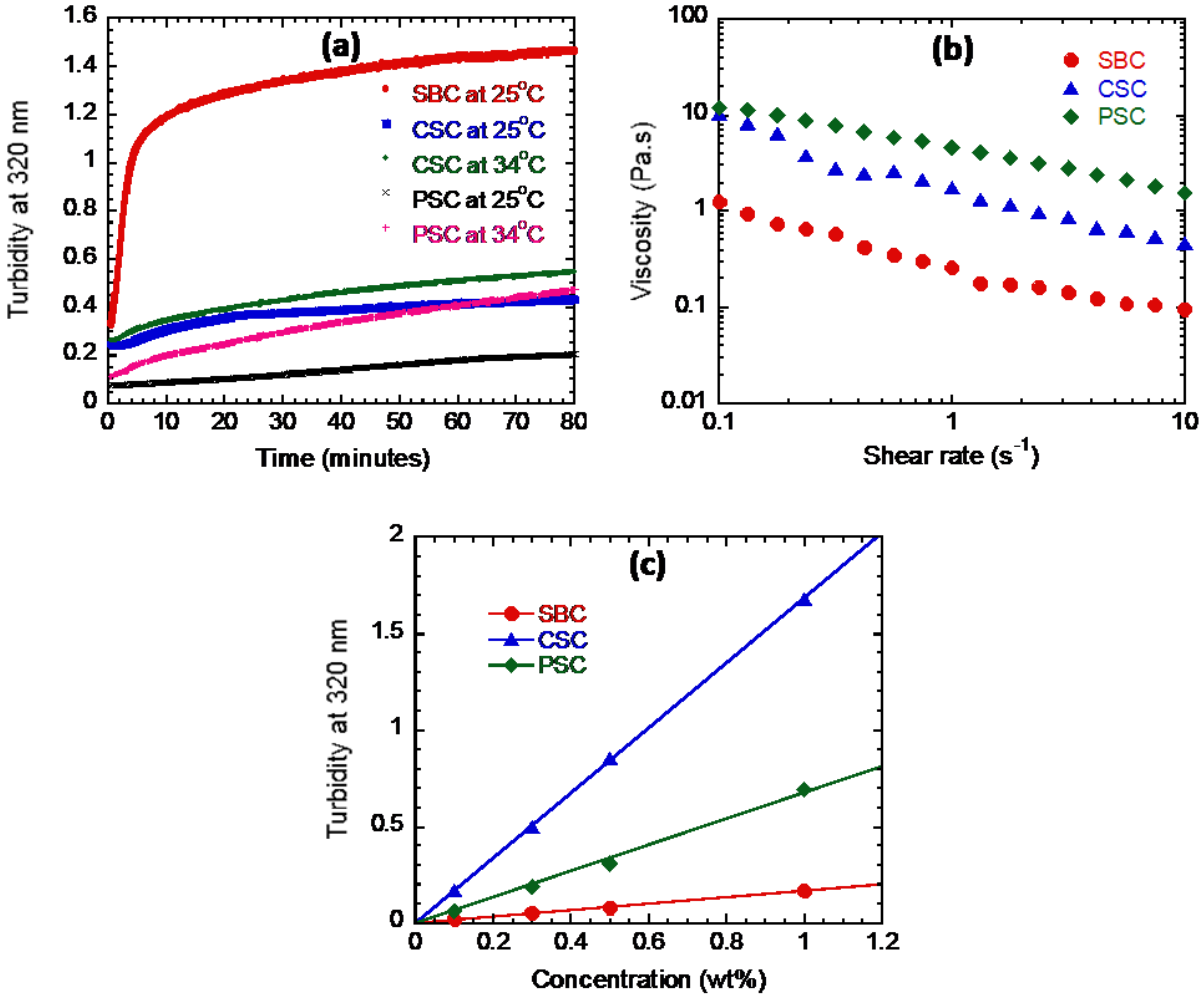


Figure 6 SEM images of **(a)** 4 wt% SBC solution, **(b)** 4 wt% SBC gel prepared at 25^{o}C, **(c)** 2 wt% CSC gel prepared at 34^{o}C, **(d)** 2 wt% PSC gel prepared at 34^{o}C. The red rectangular markers in onset samples' photographs indicate the observation region of SEM images. Lower row images are the magnified images of the corresponding upper row images.

One of the most important criteria we assumed, to develop internal stress from swelling mismatch in the RD process, is the *fast rate of bundle formation* among the rigid macromolecules with diffusing ions. Quick bundle formation causes large swelling mismatch

at the gel/sol phase boundary by solvent squeezing and therefore, can develop internal stress large enough to cause alignment of the rigid macromolecules along the gelation front line, which is frozen instantly to give the anisotropic structure. This may explain why, the fibrillogenesis rate of collagen in buffer solution must be very high to ensure the formation of aggregated structure by diffusion process and develop enough internal stress to create oriented structure. In the case of most of the conventional animal collagen, fibrillogenesis rate is very slow.[17,43,44] Zhang *et al.*[17] reported that, the fibrillogenesis rate of porcine tendon collagen is very slow compared to SBC. Figure 7(a) shows the comparative fibrillogenesis rate for SBC, CSC and PSC. At the present experimental conditions, fibrillogenesis rate of both animal collagens (CSC and PSC) is very slow, which ultimately causes the failure of creating oriented structure in CSC and PSC gels by diffusion induced gelation process. However, the fibrillogenesis rate of animal collagens (CSC and PSC) increases a bit at 34^{o}C; but may be, still far away from the required quantity of gelation rate for the creation of ordered structure.



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336 **Figure 7 (a)** Turbidity changes at 320 nm of an equal volume mixture of 0.3 wt% collagen
337 solution and 0.1 M Na-phosphate buffer (pH 7.2). The abrupt increase of turbidity of SBC
338 indicates its fast rate of *in vitro* fibril formation in the neutral buffer in comparison to CSC
339 and PSC. **(b)** The variation of dynamic viscosity (at 25°C) of acidic SBC, CSC and PSC
340 solutions with the shear rate ranges from 0.1 s^{-1} to 10 s^{-1}. **(c)** The concentration dependence of
341 turbidity at 320 nm (25°C) for acidic SBC, CSC and PSC solutions. The high turbidity of
342 CSC and PSC indicates the occurrence of fibril formation in comparison to SBC.

343

344 Additionally, the viscosity of animal collagens (CSC and PSC) in acidic solution increases
345 very rapidly with concentration, and above 2 wt% concentration, it becomes very difficult to
346 handle. For example, the viscosity of 1wt% CSC at 0.1 s^{-1} is 10.14 Pa.s and 1wt% PSC is

12.05 Pa.s, which are about one order higher than that of SBC (1.25 Pa.s) (Figure 7(b)). The high viscosity of the CSC and PSC in acidic solution is due to the formation of aggregated structure, as shown by the dramatic increase of turbidity of animal collagens at high concentration (Figure 7(c)). We speculated that these aggregated structures make it difficult to form oriented structure of collagen by swelling mismatching. In contrast, SBC solution has much lower viscosity and turbidity than CSC and PSC. This makes it possible to perform the controlled gelation of SBC at high concentration (4 wt%), close to the native tissues. In summary, fast fibrillogenesis rate, high solubility and homogeneity, and low viscosity causes extremely high degree of fiber formation, which makes the SBC very special for forming hydrogels with ordered structure.

To get better understanding about the diffusion process, we quantitatively studied the diffusion features. The small ions of neutral phosphate buffer (HPO_4^{2-}, $H_2PO_4^-$, Na^+) diffuse through the SBC solution and induce gelation. Since the fibrillogenesis rate of SBC is extremely fast and the translational motion of large triple-helix collagen molecule is very slow compared to the small buffer ions, it is expected that the gelation of SBC would be mostly controlled by the diffusion of buffer solution. This is confirmed by the linear relationship between the square of the gel layer width (distance between the periphery and the gelation front), d^2 and the gelation time, t for different concentrations of SBC as shown in Figure 8(a). The apparent diffusion coefficient, D_{app} of 0.1 M Na-phosphate buffer (pH 7.2) is calculated from Figure 8(a) using the relationship, $d^2 = 2D_{app}t$[45] and plotted against the concentrations of SBC in Figure 8(b). The values of D_{app} are in the same order with the diffusion constant of small ions in water ($D_o(HPO_4^{2-}) = 1.49 \times 10^{-5}$ cm^2s^{-1}, $D_o(H_2PO_4^-) = 1.03 \times 10^{-5}$ cm^2s^{-1}, $D_o(Na^+) = 2.53 \times 10^{-5}$ cm^2s^{-1}; calculated by using Stokes-Einstein equation[46]), and decrease linearly with increasing the concentrations of SBC. As the concentrations of

SBC increases, more buffer ions are consumed to induce fibrillogenesis and therefore, the D_{app} decreases.

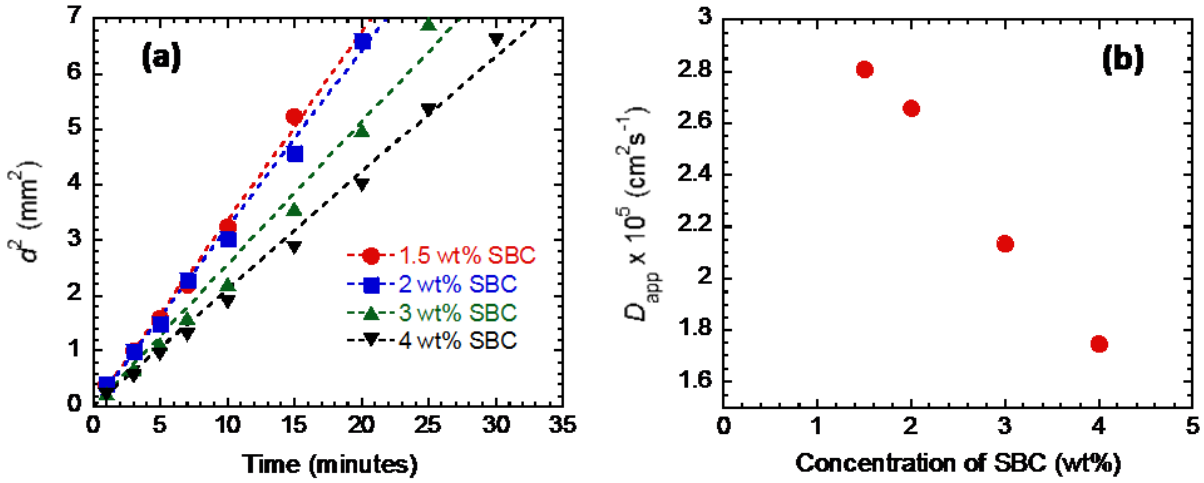
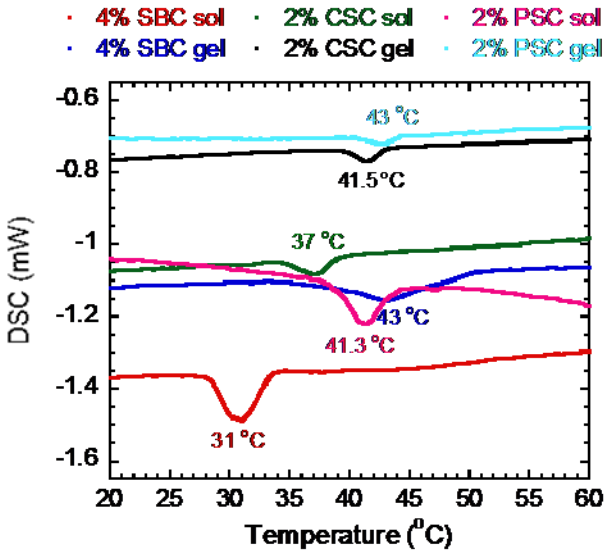


Figure 8 (a) Relationship between the square of the gel layer width (d^2) and gelation time (t) for SBC hydrogels of various concentrations. **(b)** The change in apparent diffusion coefficient, D_{app} (calculated from the slopes of (a) using the relationship, $d^2 = 2D_{app}t$) of 0.1 M Na-phosphate buffer (pH 7.2) with the concentrations of SBC.

The differential scanning calorimetry (DSC) experiments (Figure 9) show that the denaturation temperature (T_d) of SBC hydrogel rises significantly from 31^{o}C (32.9^{o}C by CD spectroscopy[17]) to 43^{o}C after the gelation, indicating the formation of thick stable fiber by diffusion induced gelation process. However in the case of CSC, T_d raises little from 37^{o}C to 41^{o}C, indicating that the further aggregation by buffer diffusion from the initial turbid solution is not so high. Similar result was observed in PSC gel. Therefore, the samples obtained from both CSC and PSC (at 25^{o}C) was very weak and it broken into fragments when we removed the cover glass of the reaction cell. On the contrary, SBC form strong gel that could be handled easily.



389

390 **Figure 9** DSC curves of SBC, CSC and PSC (both solution and hydrogel) heated at 1°C/min.

391

392 To characterize the mechanical strength of SBC hydrogel we have measured dynamic
393 modulus by applying torsion along the collagen orientation direction. The dynamic frequency
394 sweep test of 4 wt% SBC hydrogel at a constant strain of 0.2% is shown in Figure 10(a). The
395 SBC hydrogel shows a storage modulus about 15-30 kPa, which increases slightly with
396 frequency. The loss tangent is around 0.2. These results confirm that the SBC forms a soft
397 and elastic hydrogel. Figure 10(b) demonstrated that SBC gel can hang freely from the edge
398 of glass plate without any damage, indicating its self-standing ability. We found that the
399 mechanical strength and T_d value of SBC gel can be increased further by using chemical
400 cross-linker (data not shown). Since the strength of this 3D gel is sufficiently high and T_d
401 value (43°C) is well above the physiological temperature, this material would be suitable for
402 cell culture and other biomedical applications.

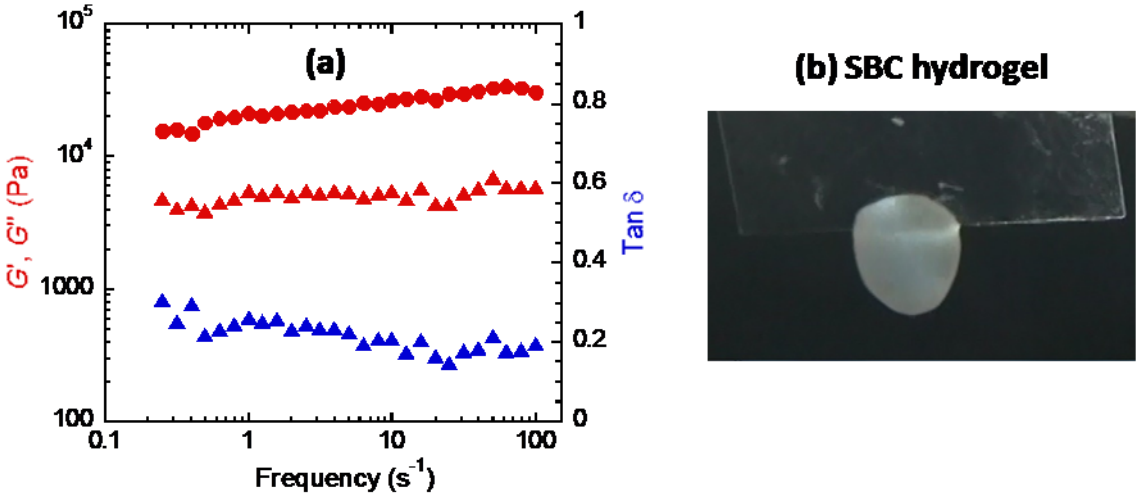


Figure 10 (a) Frequency dependence of the storage modulus (*G*'- red circle), loss modulus (*G*''- red triangle), and loss tangent (tanδ- blue triangle) of 4 wt% SBC gel at 25°C and a constant strain amplitude of 0.2%. **(b)** Free hanging of 4 wt% SBC hydrogel from the edge of glass without any damage indicates its self-standing ability.

4. CONCLUSIONS

Disk shaped physical hydrogels with concentric orientation of collagen fibrils are prepared from swim bladder collagen (SBC) of Bester sturgeon fish using a facile experimental method. SBC meets all the criteria to form oriented and self-standing hydrogel by diffusion induced gelation process. However, calf skin collagen (CSC) and porcine skin collagen (PSC) could not form any oriented structure. The high aggregated structure, slow fibrillogenesis rate, high viscosity and less homogeneity at high concentrations of animal collagens are not favorable to form ordered structure by reaction-diffusion (RD) method. On the other hand, the less aggregated structure, fast fibrillogenesis rate, and low viscosity of SBC solution favor oriented superstructure formation by the controlled diffusion of buffer. Swelling mismatch between the gel phase and the sol phase due to the quick solvent squeezing by fast fibrillogenesis process (*syneresis effect*), generates an internal tensile stress in the collagen molecules of gel phase, which assist them to align along the gel-sol interface direction to give

concentric ring-shaped orientation pattern. An anisotropic orientation gradient from periphery to center has been formed due to the change in diffusion velocity in respective regions. The denaturation temperature (T_d) of SBC hydrogel rises significantly from 31^{o}C of SBC solution to 43^{o}C due to its excellent fiber forming capacity, however; T_d of both CSC and PSC gels increase a little from that of their solutions. SBC gel has reasonably high mechanical strength (storage modulus > 15 kPa). This SBC hydrogel made from marine-based atelocollagen, having macroscopic superstructure, self-standing capability and high thermal stability, will be suitable for cell culture and other biological applications. Our study would help to understand the mechanism and requirements for creating anisotropic hydrogel by RD method. Controlling the diffusion process of neutral buffer solution through SBC solution might be able to create different orientation pattern in SBC hydrogel, which offers further opportunity for functionalization of collagen hydrogel. SBC seems to have bright prospect for creating next generation artificial bio-materials.

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Graphical abstract

Swim bladder collagen forms hydrogel with macroscopic superstructure by diffusion induced fast gelation

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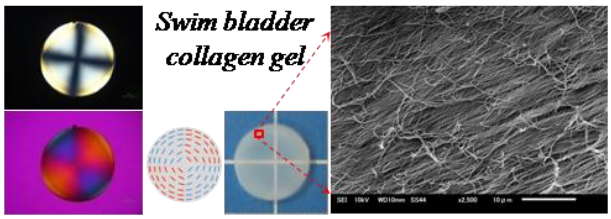

Highlights

Type I collagen extracted from swim bladder of Bester sturgeon forms oriented hydrogel with mechanical and thermal stability by diffusion induced fast gelation.