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Universality and specificity in molecular orientation in anisotropic gels prepared by diffusion method

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ABSTRACT

Molecular orientation in anisotropic gels of chitosan, Curdlan and DNA obtained by dialysis of those aqueous solutions in gelation-inducing solutions was investigated. In this diffusion method (or dialysis method), the gel formation was induced by letting small molecules diffuse in or out of the polymer solutions through the surface. For the gels of DNA and chitosan, the polymer chains aligned perpendicular to the diffusion direction. The same direction of molecular orientation was observed for the Curdlan gel prepared in the dialysis cell. On the other hand, a peculiar nature was observed for the Curdlan gel prepared in the dialysis tube: the molecular orientation was perpendicular to the diffusion direction in the outermost layer of the gel, while the orientation was parallel to the diffusion direction in the inner translucent layer. The orientation parallel to the diffusion direction is attributed to a small deformation of the inner translucent layer caused by a slight shrinkage of the central region after the gel formation. At least near the surface of the gel, the molecular orientation perpendicular to the diffusion direction is a universal characteristic for the gels prepared by the diffusion method.

1. Introduction

Biopolymer-based hydrogels have widely been investigated because of their application in food industry and potential uses in biomedical and pharmaceutical fields. In most cases, aqueous solutions of biopolymers form hydrogels by addition of various cross-linking agents such as multivalent ions. For example, alginate, a polysaccharide mainly isolated from brown algae, makes a gel when its aqueous solution is mixed with multivalent cations such as calcium ions (Donati & Paoletti, 2009). In other cases, hydrogels without any cross-linking agents can be obtained when ionic biopolymer solutions are neutralized and self-assembled fibers or aggregates are formed. For instance, collagen, the major structural protein in the extracellular matrix, self-assembles into fibrils when its acidic solution is neutralized, resulting in the gel formation without cross-linking agents (Forgacs, Newman, Hinner, Maier, & Sackmann, 2003).

Calcium-alginate gel as an example of hydrogels with cross-linking agents is often prepared by a diffusion method (or a dialysis method) (Draget, Smithrød, & Skjåk-Bræk, 2005). In the diffusion method, an aqueous solution of alginate in a dialysis tube is immersed in an aqueous solution containing calcium ions. Because of the semipermeability of the dialysis membrane, calcium ions flow through the membrane into the alginate solution and cross-link the alginate molecules, resulting in the formation of gel. The alginate gels by the diffusion method show a non-uniform alginate concentration distribution in the gels with the highest concentration at the dialysis tube and a gradual decrease in the concentration toward the center (Mørch, Donati, Strand, & Skjåk-Bræk, 2006; Skjåk-Bræk, Grasdalen, & Smithrød, 1989). Moreover, the alginate gels by the diffusion method are anisotropic showing birefringence due to the molecular orientation (Maki et al., 2009, 2011; Thiele, 1954). In the previous study we examined anisotropic structures of alginate gels in detail by birefringence and small-angle X-ray scattering (SAXS). The characteristics of the anisotropic alginate gels are summarized as follows (Maki et al., 2011). (1) Alginate molecules align circumferentially in a cylindrical gel, i.e., perpendicular to the

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direction of the calcium-ion diffusion. (2) The gel is isotropic when it is observed from its radial direction, indicating that the axis of symmetry of molecular orientation is parallel to the radial direction. (3) Rod-like structures with a radius of 6–7 nm were identified as corresponding to junction zones composed of lateral aggregates of alginate molecules by analysis of the SAXS profile of the gel, suggesting that the anisotropic structure is due to the orientation of the rod-like junction zones. (4) The gel is almost isotropic at the center and the degree of molecular orientation is higher near the surface.

Recently, Furusawa et al. (2012) reported that anisotropic collagen gels without any cross-linking agents were formed in the neutralization process. They prepared collagen gels by dialyzing acidic collagen solutions against neutral buffer solutions and studied the anisotropic gel structure by use of birefringence observation, small-angle light scattering and confocal laser scanning microscopy. It was shown that the chain orientation is perpendicular to the diffusion direction as in the case of the calcium-alginate gels, apparently indicating that the orientation direction may be universal for the diffusion-inducing gel formation.

Anisotropic gel formation by the diffusion method has also been reported for a semi-rigid synthetic polyelectrolyte (poly(2,2′-disulfonyl-4,40-benzidine terephthalamide, PBDT) cross-linked by calcium ions (Wu et al., 2011). In this case, unique molecular orientation behavior was observed. The gel was prepared in a rectangular cell consisting of two closely separated plates where one opposite sides are sealed with silicone spacers and another opposite sides are covered with dialysis membranes allowing multivalent ions to flow inside. The observation of the gel under polarizing microscope showed that the direction of molecular alignment was perpendicular to the flow of calcium ions near the surface but parallel to it near the center. This characteristic behavior implies that the orientation direction could depend on gel-forming systems.

In the previous studies, it was demonstrated that gels of various polysaccharides (Dobashi, Nobe, Yoshihara, & Yamamoto, 2004; Dobashi, Tomita, Maki, Chang, & Yamamoto, 2011; Lehtovaara, Verma, & Gu, 2012; Lin et al., 2010; Narita & Tokita, 2006; Nobe, Kuroda, et al., 2005; Nobe, Dobashi, & Yamamoto, 2005) and DNA (Furusawa, Minamisawa, Dobashi, & Yamamoto, 2007; Furusawa et al., 2010) obtained by the diffusion method were anisotropic showing birefringence. Curdlan, a microbial polysaccharide composed of β-(1,3)-glucosidic linkage, forms an anisotropic gel by immersing its alkaline solution in the dialysis tube in a copious aqueous solution of $CaCl_2$ (a dialysis tube method) (Dobashi et al., 2004; Nobe, Kuroda, et al., 2005). The similar anisotropic Curdlan gel can be also prepared by dialyzing Curdlan alkaline solution in the dialysis cell with cylindrical symmetry against aqueous $CaCl_2$ solution (a dialysis cell method) (Nobe, Dobashi, et al., 2005). The gel was sliced perpendicular to the cylindrical axis to prepare a disc-shaped specimen, and the specimen was observed under open and crossed nicols. In the observation under open nicols, the ring-shaped turbid layer was found in the gel (Dobashi et al., 2004; Nobe, Kuroda, et al., 2005). The observation under crossed nicols showed that the turbid layer and the region near the center of the gel are isotropic and the outermost region and the layer between the turbid layer and the isotropic central region are birefringent. The birefringence pattern of the anisotropic regions indicated radial or circumferential orientation of Curdlan molecules (Dobashi et al., 2004). Other kinds of gel specimens were prepared by slicing along several different planes parallel to the cylindrical axis and observed under crossed nicols, showing that the axis of symmetry of the orientation was along the radial direction (Dobashi et al., 2004).[3]

Anisotropic DNA gels induced by the diffusion method have been prepared in a different experimental setup (Furusawa et al., 2007, 2010). When aqueous solution of DNA sandwiched by a pair of circular cover glasses was immersed in a solution containing multivalent metal ions such as Co^{2+}, Cu^{2+}, and Al^{3+}, a gel film was formed due to ionic cross-linking between anionic DNA and multivalent cations. The DNA solution near the edge of the cover glass forms gel by the diffusion of metal ions immediately after the immersion and the gel membrane formed at the edge of the cover glass would act as a dialysis membrane (Furusawa et al., 2007). The gel film observed under crossed nicols exhibited a 'Maltese cross' birefringence pattern similar to the anisotropic alginate gels by the dialysis method, indicating radial or circumferential orientation of DNA molecules. A gel film of chitosan, a random copolymer composed of β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine, has been prepared by the similar method (Dobashi et al., 2011). Chitosan dissolved in an aqueous acetic acid solution was sandwiched by a pair of cover glasses, followed by the immersion in a NaOH aqueous solution. In the neutralization process, chitosan molecules are insolubilized and form aggregates, followed by the formation of gel (Ladet, David, & Domard, 2008; Schatz, Pichot, Delair, Viton, & Domard, 2003). The obtained gel film exhibited a birefringence pattern similar to those of the other polysaccharides and DNA. The anisotropic gels of DNA and chitosan are hydrogels with and without cross-linking agents, respectively. The situation for the anisotropic Curdlan gel is rather complicated because Curdlan can form both types of gels: the gel formed by the cross-link with multivalent cations and that formed by the molecular aggregation in the neutralization process. In the dialysis process of Curdlan alkaline solution in $CaCl_2$ solution, the diffusion of Ca^{2+} into the Curdlan solution and the diffusion of OH^- out of the Curdlan solution occur simultaneously, inducing the gelation via cross-linking with Ca^{2+} and via the molecular aggregation. Because Ca^{2+} ions encounter Curdlan molecules at higher pH at the outer region of the dialysis tube and at lower pH at the inner region, respectively, it is expected that the Ca^{2+} cross-linked gel is formed near the surface of the cylindrical gel and the aggregation-induced gel is formed near the center.

In the previous studies, the direction of the molecular orientation has not exactly been determined for the anisotropic gels of Curdlan, chitosan and DNA. In the present study, the molecular alignment in these biopolymer gels by the diffusion method is investigated in order to clarify universal and specific aspects in the molecular orientation in the diffusion-induced gel formation.

2. Experimental

DNA purified from salmon milt with ca. 10 kbp was provided by Nippon Chemical Feed Co. Ltd., Japan. Chitosan (chitosan300, deacetylation ratio > 80%) with average molecular weight $M_w = 1.1 \times 10^6$ was purchased from Wako Pure Chemical Industries Ltd., Japan. Two kinds of Curdlan samples were used: one with $M_v = 5.9 \times 10^5$ was from Wako Pure Chemical Industries Ltd. (CD1) and the other with $M_v = 2.1 \times 10^6$ was from Takeda-Kirin Foods Corp., Japan (CD2). These samples were used without further purification. Aluminum chloride, sodium tetra-borate, sodium hydroxide, calcium chloride and acetic acid of reagent grade were used. Milli-Q water was used for solvent.

In the preparation of the anisotropic gel of DNA (Furusawa et al., 2007, 2010; Furusawa, Minamisawa, Dobashi, & Yamamoto, 2009), DNA was dissolved in 20 mM sodium tetra-borate solution (pH 9.2)

[3] In the previous study (Dobashi et al., 2004), the authors concluded that Curdlan molecules aligned radially in the cylindrical gel. However, their experiments indicate that the axis of symmetry of the orientation is along the radial direction, but whether the direction of orientation is radial or circumferential cannot be identified from their data.

to be a solution of the concentration of 1.0 wt%. Then, 200 μL of the solution was sandwiched by a pair of circular cover glasses with the diameter of 15 mm, and then immersed into a copious amount of aqueous solution containing aluminum chloride at 100 mM to obtain the DNA gel. For the anisotropic gel of chitosan, a chitosan solution of the concentration 2.0 wt% in 2.0 wt% aqueous acetic acid solution was prepared and 200 μL of the solution was put between a pair of cover glasses with the diameter of 15 mm and immersed in a large amount of 0.3 M NaOH solution (Dobashi et al., 2011; Yamamoto, Tomita, Maki, & Dobashi, 2010). The gel film of DNA or chitosan was observed under a pair of polarizers at crossed nicols or a pair of circular polarizers (Supplementary data, S1). The experimental setup of the circular polarizers has been used as in the previous study (Maki et al., 2011).

Two slightly different methods, i.e., a dialysis tube method yielding cylindrical gels and a dialysis cell method yielding disc-shaped gels, were used to obtain the anisotropic gels of Curdlan (Supplementary data, S2). In the dialysis tube method (Dobashi et al., 2004; Maki et al., 2011; Nobe, Kuroda, et al., 2005), Curdlan was dissolved in 0.3 M NaOH solution at the concentration of 3.0, 4.0 or 5.0 wt% and the solution (ca. 50 g) was poured into a seamless cellulose tube with the diameter of 25 or 28 mm (Sanko Pure Chemical Co. Ltd., Japan) and then was dialyzed against into 1 L of aqueous solution of $CaCl_2$ at 8 g/dL. A disc-shaped specimen was prepared by slicing the cylindrical Curdlan gel perpendicular to the axis of the cylinder and the specimen was observed in the same way as for DNA and chitosan specimens. In the dialysis cell method (Maki et al., 2009; Nobe, Dobashi, et al., 2005), we used a cell consisting of two pieces of disc-shaped plexiglass of ca. 30 mm in diameter placed face-to-face at a distance of ca. 1 mm and a seamless cellulose tube of 28 mm in diameter (Sanko Pure Chemical Co. Ltd.) covering the side of the discs. Curdlan solution at 5.0 wt% in 0.3 M NaOH was poured into the cell through a hole of the diameter 3 mm on the upper disc. After the hole had been tightly sealed, the cell was immersed in 500 mL of $CaCl_2$ solution of 8 g/dL. The obtained disc-shaped gel was observed in the same way as the other gels.

For the determination of the sign of birefringence for DNA, chitosan and Curdlan, fibers of these polymers were prepared and observed under a pair of circular polarizers. The fibers were spun from the solutions and put into ethanol as a coagulation bath, followed by drying in air.

In order to examine the triple-helix formation in the anisotropic Curdlan gels, small-angle X-ray scattering (SAXS) measurements were performed using the focusing small-angle diffractometer installed at the beamline 15 A of the Photon Factory (Amemiya, Wakabayashi, & Itoh, 1998) at the High Energy Acceleration Research Organization in Tsukuba, Japan. Intense synchrotron radiation X-rays with wavelengths of 1.5 Å emitted from the positron storage ring were collimated with doubly focusing optics and used for the SAXS measurements. A disc-shaped specimen of the Curdlan gel by the dialysis tube method with the thickness of 1 mm was prepared in the same way as that for the birefringence observation. The specimen was set into a sample holder for SAXS measurements, and X-rays were incident on a desired position of the specimen from the direction perpendicular to the disc. The two-dimensional SAXS pattern was recorded with the area detector consisting of an image intensifier coupled to a CCD camera (model C7300, Hamamatsu Photonics, Japan). The spatial distortion and non-uniformity of the response of the detector were corrected following Ito, Kamikubo, Yagi, and Amemiya (2005). The background scattered intensity measured under the same experimental condition was subtracted from the scattered intensity of the sample. A circular-average was performed for the two-dimensional SAXS images in order to obtain one-dimensional intensity profiles with a high S/N as functions of scattering vector length q ($q = 4\pi \sin\theta/\lambda$ where 2θ and λ are the scattering angle and the wavelength of X-rays (1.5 Å), respectively).

Positional dependence of Young's modulus E of the Curdlan gel was measured by means of the indentation method. A disc-shaped specimen of the gel with the thickness of 5 mm was prepared from the cylindrical gel by the dialysis cell method. The specimen was indented perpendicularly to the disc at each position by a cylindrical stainless steel probe with the diameter $a = 0.98$ mm. The indentation force f as a function of time t after applying instant step deformation $\delta = 0.3$ mm to the gel was measured by a digital multimeter receiving a voltage signal V from a load cell (LC4001-G120, A&D, Japan) connected with the probe using a calibration equation of V (mV) = 2.108 f (N). Young's modulus was estimated using the Hertz model as

$$f = \frac{2E}{1-\nu^2} a\delta \quad (1)$$

where the Poisson's ratio ν was assumed to be 0.5.

All experiments were carried out at the temperature of 25 °C.

3. Results and discussion

Fig. 1(a)–(c) shows the photographs of diffusion-set gels of DNA, chitosan and Curdlan, respectively, observed under crossed nicols. Here the Curdlan gel is prepared by the dialysis cell method. All gels exhibited a birefringence pattern with crossed dark lines ('Maltese cross' pattern), indicating either radial or circumferential orientation of molecules. Fig. 1(d)–(f) shows the photographs of the same gels as in Fig. 1(a)–(c), respectively, observed under a pair of circular polarizers. Arrows in Fig. 1(d)–(f) represent the direction of the slow axis of the circular polarizers. In the observation of a birefringent sample under a pair of circular polarizers, it appears blue (orange) colored when a component of the refractive indices for the direction parallel to the slow axis of the circular polarizers is larger (smaller) than that for the direction perpendicular to the slow axis. A pattern of blue and orange sectors for the chitosan gel was the same as the color pattern for the alginate gel studied in the previous study (Maki et al., 2011) and this pattern indicates that the circumferential component of the refractive indices is larger than the radial component for chitosan. However, patterns for the DNA and Curdlan gels show an opposite layout of the blue and orange regions. These results indicate that the circumferential component of refractive indices is smaller than the radial component for DNA and Curdlan gels. On the other hand, the sign of the intrinsic birefringence for chitosan, DNA and Curdlan molecules was determined from the birefringence observation of their fibers: the intrinsic birefringence was positive for chitosan and negative for DNA and Curdlan. Thus, the direction of molecular orientation was circumferential, i.e., perpendicular to the diffusion direction, for the gels of chitosan, DNA and Curdlan. This orientation direction is the same as that for the diffusion-set gels studied previously: the anisotropic gels of alginate (Maki et al., 2011) and collagen (Furusawa et al., 2012). Thus, the molecular orientation perpendicular to the diffusion direction is a universal characteristic for the gels prepared by the diffusion method irrespective of whether or not the gel formation was induced by any cross-linking agents. In the previous studies (Dobashi et al., 2011; Furusawa et al., 2007, 2009, 2010; Lin et al., 2010; Maki et al., 2009; Nobe, Dobashi, et al., 2005; Yamamoto et al., 2010), the gelation process by the diffusion method was represented by the movement of a sharp gelling front which separates a gel phase from a sol phase. The observed sharp gelling front would be due to an abrupt decrease in chemical potential of the polymer from the sol phase toward the gel phase (T. Yamamoto, private communication), inducing molecular orientation along the gel front, i.e., perpendicular to the diffusion direction. In the case of the gelation via cross-linking, incorporation of flexible chains in the sol phase into the gel region would occur as follows (Woelki & Kohler, 2003). When polymer segments collide with the

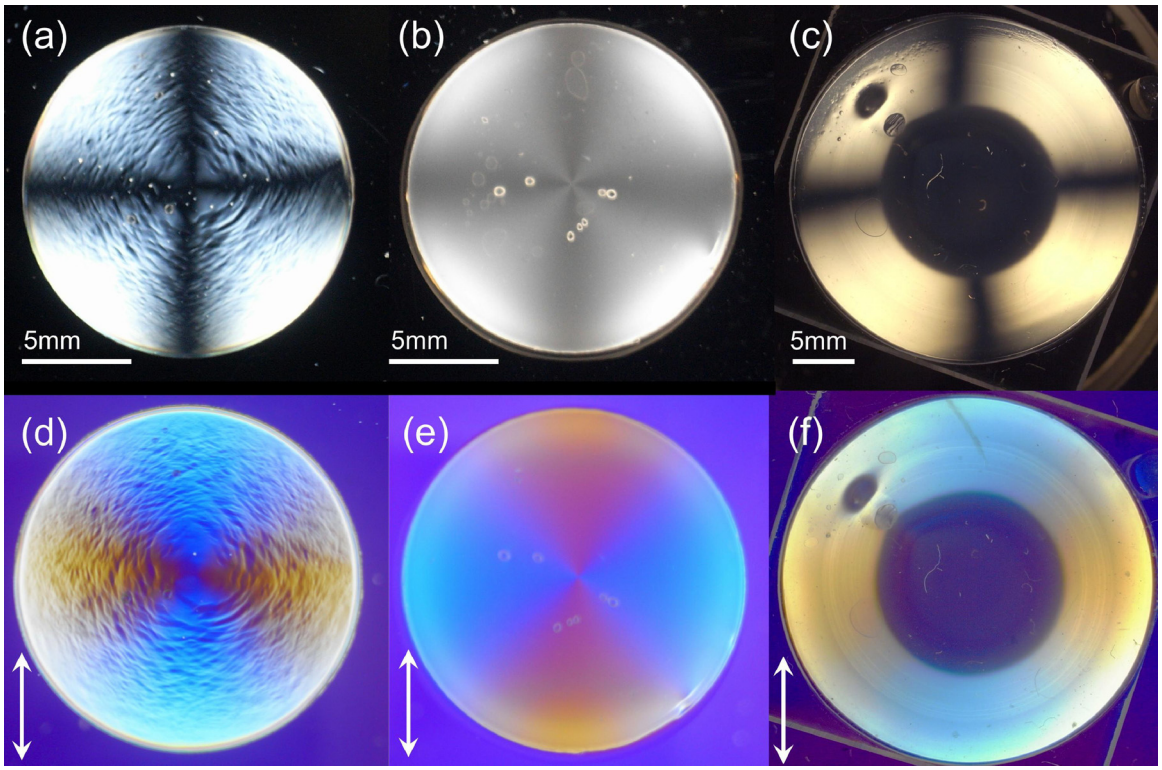

Fig. 1. Photographs of anisotropic gels of DNA ((a) and (d)), chitosan ((b) and (e)) and Curdlan ((c) and (f)). The photographs (a)–(c) were obtained under crossed nicols and (d)–(f) were obtained under a pair of circular polarizers. The Curdlan gel was prepared from alkaline solution of CD1 at 5.0 wt% by the dialysis cell method. Arrows represent the direction of the slow axis of the circular polarizers.

gel front, the segments could be bound on the front by immediate formation of cross-linking owing to the abrupt decrease in chemical potential of the polymer. Due to the fixation of the segments on the front, the motion of the remaining segments toward the sol phase is restricted and the average direction of the segment motion tends to be toward the gel phase. As a result, extension of the polymer chains parallel to the gel front and contraction of the chains perpendicular to it occur. If a similar mechanism works for more rigid polymer chains, a propensity of the unbound segments to move toward the gel phase results in orientation of the whole polymer chains along the gel front, as was suggested by Wu et al. (2011). In the case of the gelation via molecular aggregation, a fibril of aggregated polymer chains would be more stable when they aligned along to the gel front due to the decrease in the chemical potential. In addition, the aligned fibrils would facilitate a further fibril growth in the direction parallel to the gel front.

For the more critical discussion of the orientation direction of Curdlan molecules, an effect of molecular conformation on the sign of birefringence may have to be taken into account. In the present study, Curdlan molecules were dissolved in 0.3 M NaOH, and then the alkaline solution of Curdlan was dialyzed against the aqueous $CaCl_2$ solution. Thus, pH value of the solution decreases gradually in the dialysis process. Ogawa, Watanabe, Tsurugi, and Ono (1972) studied the conformational behavior of Curdlan molecules in a dilute solution as a function of NaOH concentration by the measurements of optical rotation, flow birefringence and viscosity, and suggested that the conformational transition occurs in the region of NaOH concentrations between 0.19 and 0.24 M from an ordered helical conformation at lower NaOH concentrations to a random coil at higher NaOH concentrations. Saito, Ohki, and Sasaki (1977) measured ^{13}C NMR of alkaline Curdlan solutions and observed the helix-to-coil transition at the NaOH concentrations between 0.19 and 0.22 M. Therefore, in the anisotropic gel formation process, the conformation of Curdlan molecules could change from a random coil to a helical structure. In the previous study on the gelation process of Curdlan by the diffusion method, pH of the inner Curdlan solution was measured as a function of time during the dialysis. pH of the inner solution decreased below 13.3 corresponding to the NaOH concentration of 0.2 M immediately after the start of the dialysis (Nobe, Dobashi, et al., 2005). This result suggests that the Curdlan molecules in the diffusion set gels assume a helical structure. In order to examine the conformation of Curdlan, small-angle X-ray scattering (SAXS) was measured for the anisotropic gel of Curdlan. Fig. 2 shows the SAXS intensity data of the Curdlan gel prepared by the dialysis tube method which were obtained at different positions from the center of the gel set at 0. In Fig. 2, the SAXS intensity profiles are plotted against the scattering vector length q and the plots at the different positions are shifted vertically for clarity. A broad peak is observed around at $q = 0.37$ Å^{-1} for each position of the gel and the corresponding Bragg spacing defined as $2\pi/q$ is ~17 Å. This value is close to the spacing value of 17.4 Å for the Curdlan gel obtained by neutralization of the alkaline solution of Curdlan (Kanzawa, Harada, Koreeda, Harada, & Okuyama, 1989), indicating that the Curdlan molecules in the diffusion-set gel have helical conformation as found at low concentrations of NaOH. As described in Section 2, the Curdlan fiber used for the determination of the birefringence sign was spun from the Curdlan solution of 0.3 M NaOH, where the molecular conformation of Curdlan is a random coil. For the determination of the birefringence sign of Curdlan in helical conformation, an oriented film was prepared from a neutralized gel of Curdlan as follows. Curdlan in alkaline solution was dialyzed in pure water and the obtained neutralized gel was then dried under a compressive load, resulting in the oriented film. It was reported previously that the neutralized gel obtained by the dialysis of Curdlan in pure water was isotropic (Sato, Nobe, Dobashi, Yamamoto, & Konno, 2005). The

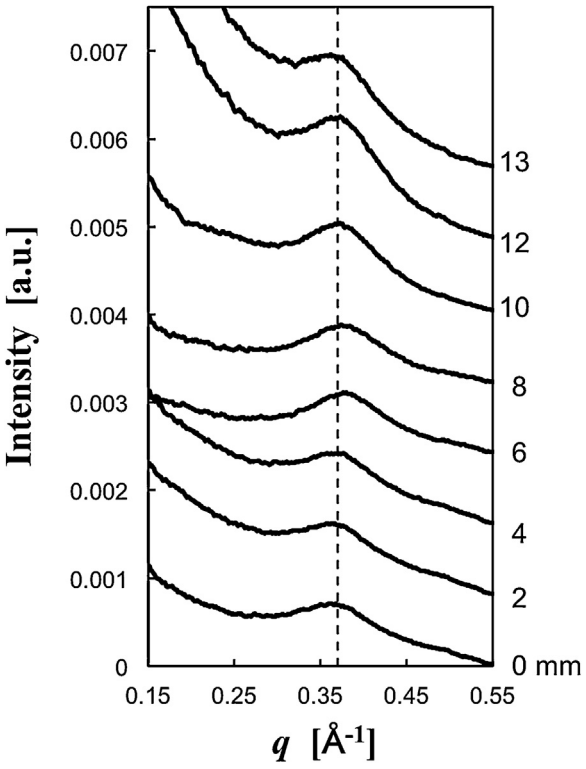

Fig. 2. SAXS intensity profiles of the Curdlan gel at different positions from the center of the gel. Each plot is shifted vertically by 0.0008 units for clarity. The Curdlan gel was prepared from alkaline solution of CD1 at 5.0 wt% by the dialysis tube method with φ = 28 mm cellulose tube.

Curdlan molecules should assume helical structure in this neutralized gel and the molecular orientation in the film is induced by the applied load. The observation of the oriented film under a pair of circular polarizers indicated negative birefringence of Curdlan in helical conformation. This result is consistent with a negative flow birefringence in a dilute solution at low NaOH concentrations (Ogawa et al., 1972). The negative birefringence of Curdlan in helical conformation confirmed the circumferential orientation of Curdlan molecules in the anisotropic gel by the diffusion method as mentioned above.

The above results indicate that the direction of molecular orientation in anisotropic gels by the diffusion method is perpendicular to the diffusion direction. However, a different behavior was observed for the Curdlan gel prepared by the dialysis tube method. Fig. 3(a)–(c) shows the photographs of the Curdlan gel prepared by the dialysis tube method observed under natural light, crossed nicols and a pair of circular polarizers, respectively. Fig. 3(a) shows that the Curdlan gel consists of several layers: the outermost translucent layer (①), the outer turbid layer (②), the inner translucent (③) layer and the central turbid layer (④) (Dobashi et al., 2004),[4] from the surface to the center of the gel. In Fig. 3(b), the outermost and inner translucent layers exhibit birefringence and the outer and central turbid layers are isotropic. In Fig. 3(c), the pattern of blue and orange regions in the outermost translucent layer appears similar to that of Fig. 1(f). However, closer inspection of Fig. 3(c) shows that there is a vague color pattern of the opposite layout to that of Fig. 1(f) in the inner translucent layer. In order to examine this appearance more clearly, the intensity profiles of the RGB components of Fig. 3(c) were investigated. Fig. 4 shows the intensity profiles of the RGB components along the lines 1 and 2 in the photograph under a pair of circular polarizers. Arrows in Fig. 4 represent the local maxima or local minima in intensity of the red or blue component, corresponding to the color pattern different from that in the outer translucent layer of the gel. In Fig. 4, the positions of the outer turbid layer and the central turbid layer in Fig. 3(a) are represented as the shaded regions, indicating that the inversion of the color pattern occurs just between the inner translucent layer and the central turbid layer. There are two possible reasons for the inversion of the color pattern. (1) Molecular alignment along the radial direction and (2) effect of the form birefringence (Doi & Edwards, 1986) due to the circumferential deformation of domains in which polymer concentration is different from that of the outside matrix. In the case of (2), evaporation of the solvent would cancel out the difference in the dielectric constants of the domains and the matrix, resulting in the change of the color pattern. When the disc-shaped gel specimen was dried to form a film and the film was observed under a pair of circular polarizers, no change in the color patterns was seen both in the outer translucent layer and in the inner translucent layer. This observation indicates that the inversion of the color pattern is due to the molecular orientation along the radial direction, i.e., the diffusion direction of Ca^{2+} ions.

In order to clarify the mechanism of the distinctive molecular orientation in the Curdlan gel prepared by the dialysis tube method, the gelation process was observed in detail. The samples prepared in dialysis tubes were taken out of the dialysis solution at different dialysis time periods and the cross section of the gel samples was observed under natural light and a pair of circular polarizers. The observation was made at intervals of 1 h for the samples of the dialysis time ranging from 1 to 24 h (Supplementary data, S3). Fig. 5 shows some of the obtained photographs. The photographs under natural light in the upper row show that the gel front moves from the surface of the tube and reaches the center in 6 h. At this time period, the gel formed at the center is rather transparent. Subsequently, a ring-shaped turbid layer appears just at the inside of the inner translucent layer and it grows toward the center, finally forming the central turbid region. The formation of the central turbid layer has not been reported in the previous studies (Dobashi et al., 2004) because of the short dialysis time periods. The photographs in the lower row in Fig. 5 are those observed under a pair of circular polarizers. The outermost translucent layer shows that the molecular orientation is circumferential independent of the dialysis time period. On the other hand, it was shown that the circumferential orientation in the inner translucent layer at the dialysis time of 6 h changes into the radial orientation at the dialysis time of 20 h. The inversion of the orientation direction was not observed for the dialysis time periods shorter than 13 h, and a sign of the inversion started to appear for the dialysis time periods between 14 and 17 h, and the inversion became completed for the dialysis time periods longer than 18 h (Supplementary data, S3). The dialysis time required for the inversion of the orientation in the inner translucent layer was comparable to that for the completion of the central turbid layer formation. The observed molecular orientation perpendicular to the diffusion direction near the surface and parallel to it near the center as shown in Fig. 3(c) is similar to that for the anisotropic PBDT gel (Wu et al., 2011). For the PBDT gel, it was suggested that the molecular orientation parallel to the diffusion direction near the center is caused by longitudinal diffusion of PBDT molecules in the sol phase from the central region toward the gel front. This is not the case for the Curdlan gel because the inversion of the orientation occurs after the gel formation and thus another explanation is needed.

As was mentioned in Introduction, Curdlan gels can be formed from its alkaline solutions both by addition of multivalent ions

[4] In Fig. 1 of the paper of Dobashi et al. (2004), the central region of the Curdlan gel seemed to appear rather transparent and the central turbid layer as in Fig. 3(a) was not observed. This difference is attributed to a shorter dialysis time period of the gel formation in their study, as shown later.

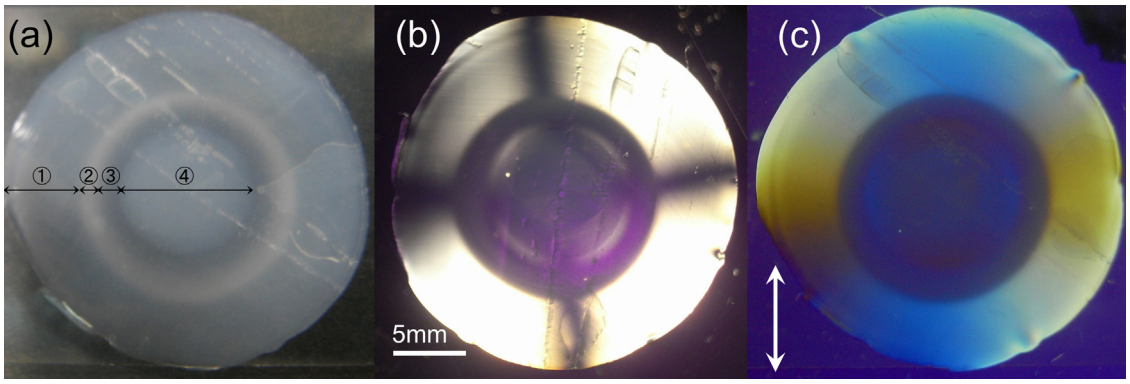

Fig. 3. Photographs of the anisotropic gels of Curdlan prepared from the alkaline solution of CD1 at 5.0 wt% by the dialysis tube method with φ25 mm cellulose tube. The photographs (a), (b) and (c) were obtained under open nicols, crossed nicols and a pair of circular polarizers, respectively. Arrow in (c) represents the direction of the slow axis of the circular polarizers.

and in the neutralization process and it is suggested that the Ca^{2+} cross-linked gel is formed near the surface and the neutralization-induced gel is formed near the center in the dialysis process of Curdlan alkaline solution in $CaCl_2$ solution. If this is the case, the transparent gel formed first at the center of the tube could correspond to the neutralization-induced gel, and the subsequent growth of the turbid layer (Fig. 5) at the center of the tube could be due to the change in the gel structure caused by the increase in the Ca^{2+} concentration. Although the details of the structural change remain to be clarified, the formation of crosslinks by Ca^{2+} or microphase separation could occur in the neutralization-induced gel due to the increase in the Ca^{2+} concentration.

In order to examine the effect of the growth of the inner turbid layer on the physical property of the gel, positional dependence of Young's modulus E was measured for the Curdlan gels of the different dialysis time periods. Fig. 6(a) shows the equilibrium modulus E_{eq} as a function of the distance x from the center of the gel specimen for the gels of dialysis time period of 7 h and 24 h. A typical relaxation modulus $E(t)$ after the small indentation is shown in the inset of Fig. 6(a) where the equilibrium modulus E_{eq} can be estimated as the value of $E(t)$ at $t = 1$ min. Fig. 6(b) shows the grayscale values of the photographs under natural light for the gels of 7 h and 24 h at different positions, where the maximum grayscale value of 255 corresponds to bright white. In Fig. 6(a), there are a local minimum and a local maximum in E_{eq} near the outer edge of the outer turbid layer and the inner translucent layer, respectively. The local minimum in E_{eq} could be relevant to the previously reported fact that the outermost translucent layer was easily peeled off at the

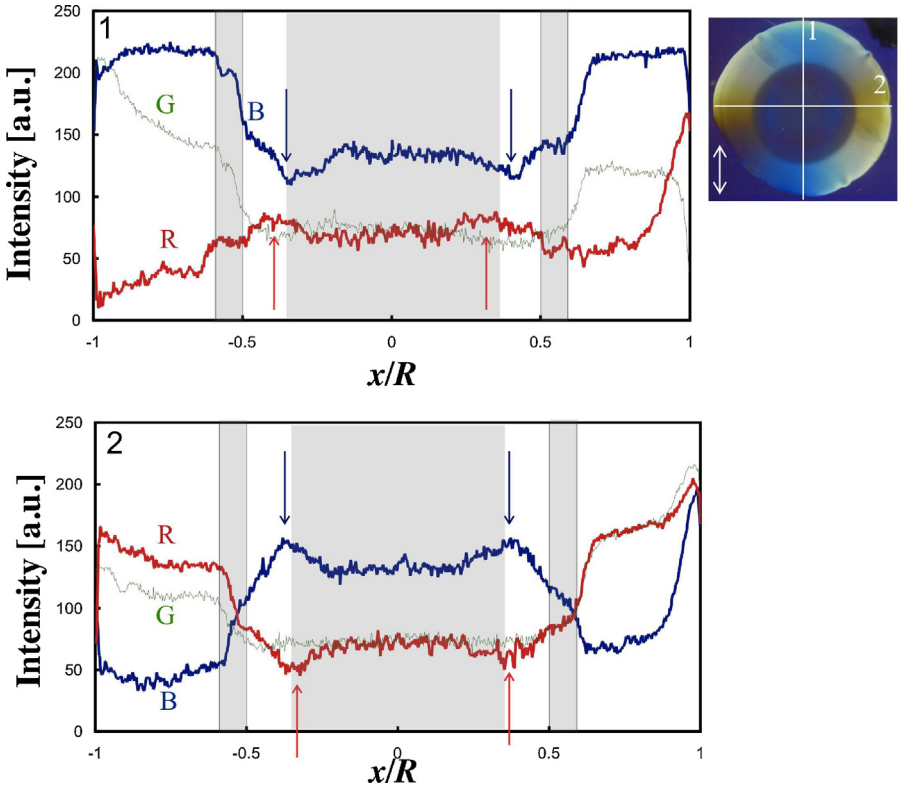

Fig. 4. Intensity profiles of the RGB components along the lines 1 and 2 in the photograph of Curdlan gel (upper right) under a pair of circular polarizers. The abscissa is the ratio of the radius of the gel region at the distance x from the center of the gel to the radius R of the whole gel. Arrows show the local maxima or the local minima in intensity of the red or blue component and the shaded regions represent the turbid layers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

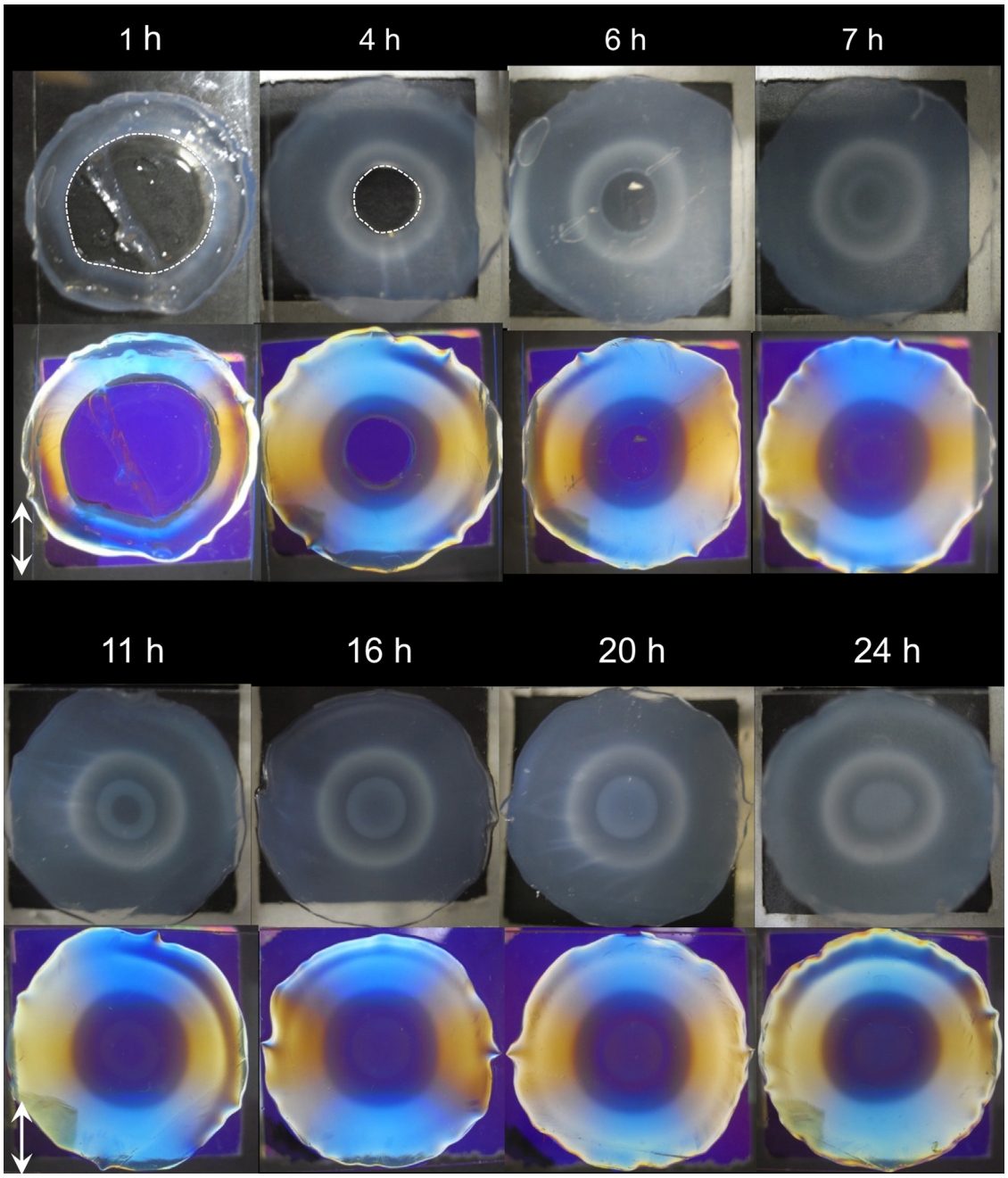

Fig. 5. Photographs of the anisotropic gels of Curdlan for different dialysis time periods observed under natural light (upper rows) and under a pair of circular polarizers (lower rows). A dotted line in the photograph for the dialysis time period of 1 h and 4 h represents the gel front line. The Curdlan gels were prepared from alkaline solutions of CD2 at 3.0 wt% by the dialysis tube method with φ28 mm cellulose tube. Arrows represent the direction of the slow axis of circular polarizers.

boundary of the outermost layer and the outer turbid layer (Dobashi et al., 2004). The behavior of E_{eq} at the outer layers is almost independent of the dialysis time but the value of E_{eq} at the central region increases with the dialysis time. E_{eq} in the central region increases with the growth of the inner turbid layer. As shown in Fig. 6(b), the central region is almost transparent (lower grayscale values) for dialysis of 7 h whereas the central turbid region has been formed (higher grayscale values) for dialysis of 24 h.

Fig. 6 clearly shows the significant change in the mechanical property of the gel at the central region after the gelation has finished. If there is a slight shrinkage of the central region due to the change in the gel structure, the adjacent inner translucent layer could be deformed, resulting in the emergence of the radial molecular orientation. To verify this view, a relative size r/R of the central region in the photographs in Fig. 5 was plotted against the dialysis time in Fig. 7, where r and R are a radius of the central gel region and that of the whole gel, respectively. Although the shrinkage seems not significant in Fig. 5, Fig. 7 shows that the size of the central region tends to decrease slightly (at most ca. 10%) with the dialysis time. In order to estimate the deformation of the inner translucent gel layer required for the inversion of the birefringence sign, a portion (ca. 2 mm on a side) of the inner translucent layer cut from the gel for dialysis of 7 h was deformed with a tweezers and observed under crossed nicols and a pair of circular polarizers. At this dialysis time, the inner translucent layer had a circumferential molecular orientation. As the gel portion was elongated in the radial direction, transmitted light intensity under the crossed nicols was changed. In Fig. 8 the average grayscale values of the

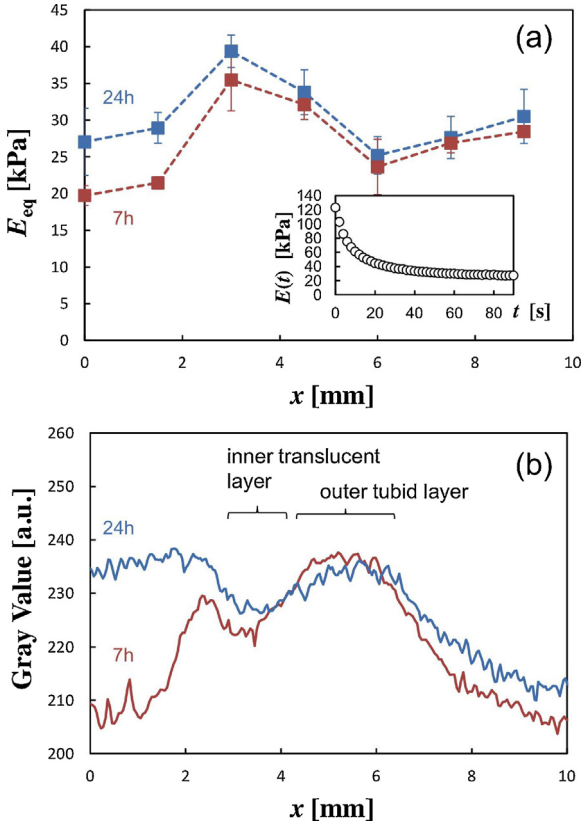

Fig. 6. Equilibrium Young's moduli E_{eq} (a) and grayscale values of the photographs under natural light (b) as a function of the distance from the center of the Curdlan gels at the dialysis time periods of 7 h (red) and 24 h (blue), respectively. The inset of (a) shows the relaxation modulus $E(t)$ after the small indentation at the center of the gel of 24 h dialysis. The Curdlan gels were prepared from alkaline solutions of CD2 at 3.0 wt% by the dialysis tube method with φ28 mm cellulose tube. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

photographs of the gel portion under crossed nicols are plotted against the strain $\varepsilon = (l - l_0)/l_0$, where l and l_0 represent the length of the gel in the longitudinal direction after and before the deformation, respectively. The grayscale value slightly decreased with ε, followed by the gradual increase, representing the change in the molecular orientation. The inversion of the birefringence sign was more obvious in the photographs under a pair of circular polarizers (the inset of Fig. 8). The color of the gel changed from orange to blue

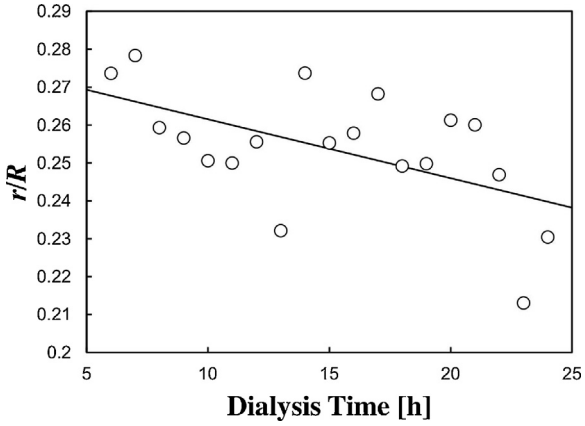

Fig. 7. Plot of the relative size r/R of the central region in the photographs of Curdlan gel under natural light in Fig. 5 against the dialysis time. r and R are radius of the central gel region and that of the whole gel, respectively.

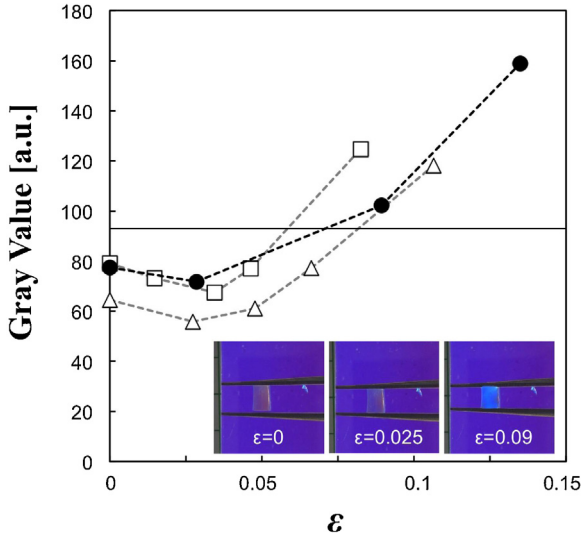

Fig. 8. Plots of the grayscale values of the photographs of a portion of the inner translucent layer in the Curdlan gel at the dialysis of 7 h observed under crossed nicols against the strain ε of the gel portion. Different symbols are used to distinguish the samples used in the experiment. A horizontal line represents the grayscale value of an undeformed gel portion for the Curdlan gel of 24 h dialysis. The Curdlan gel was prepared from alkaline solutions of CD2 at 3.0 wt% by the dialysis tube method with φ = 28 mm cellulose tube. The inset shows the photographs observed under a pair of circular polarizers for the gel portion corresponding to the data represented by filled circles.

as ε increased. As shown in Fig. 8, the measurements were carried out for three samples and similar trends were observed; deviations in the grayscale values could be attributed to small differences in the size and shape of the gel portions. In Fig. 8, the grayscale value of an undeformed gel portion cut from the inner translucent gel layer for the dialysis time period of 24 h is shown for the comparison. For the inner translucent layer of the dialysis of 7 h, a small deformation of ε of ~0.1 is suffient for the birefringence sign to invert and the birefringence to reach the comparable value at the dialysis of 24 h. Thus, it is suggested that the radial orientation of molecules in the inner translucent layer of the Curdlan gel by the dialysis tube method is attributed to a small deformation of the layer caused by a slight shrinkage of the central gel region. Although the orientation direction of the polymer chains is apparently similar to that for the PBDT gel, the inversion of the molecular orientation observed for the Curdlan gel is a specific aspect in the anisotropic gel formation because the mechanism of the inversion is different from the PBDT gel. It should be noted that the inversion of the molecular orientation in the inner translucent layer was not observed for the Curdlan gel prepared by the dialysis cell method as shown in Fig. 1, although the growth of the inner turbid layer was also observed for the dialysis cell method. The photograph in Fig. 1 was obtained at the dialysis time period of 18 h and the inversion of birefringence was not observed yet even after long dialysis of 47 h (data not shown). In the dialysis cell method, the shrinkage of the central gel region, as is observed in the dialysis tube method, may be prevented because the gel shape is more constrained by the wall of the dialysis cell.

4. Conclusion

Dialysis of the solutions of DNA, chitosan and Curdlan in the gel-inducing solutions induced the orientation of polymer chains, resulting in the formation of anisotropic gels. The molecular orientation perpendicular to the diffusion direction was confirmed for the gels of DNA and chitosan, which was consistent with the results for gels of alginate and collagen by the diffusion method

(Furusawa et al., 2012; Maki et al., 2011). The anisotropic gel of Curdlan showed a multi-layered structure with outermost translucent layer, outer turbid layer, inner translucent layer and central turbid region. The direction of molecular orientation in the Curdlan gel prepared by the dialysis cell method was same as that in anisotropic DNA and chitosan gels. However, the molecular orientation of the Curdlan gel prepared by the dialysis tube method was different in the outermost and inner translucent layers: perpendicular to the diffusion direction in the outermost translucent layer and parallel to it in the inner translucent layer. The latter molecular orientation was attributed to a small deformation of the inner translucent layer caused by a slight shrinkage of the central region after the gel formation. At least near the surface of the gel, the molecular orientation perpendicular to the diffusion direction is a universal characteristic for the gels prepared by the diffusion method irrespective of whether or not the gel formation was induced by any cross-linking agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.03.012.

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