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Immobilization of laminin peptide in molecularly aligned chitosan by covalent bonding

Atsushi Matsuda[a,b,*], Hisatoshi Kobayashi[a], Soichiro Itoh[c], Kazunori Kataoka[a,d], Junzo Tanaka[a,e]

[a]*Biomaterials Research Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*
[b]*Japan Society for the Promotion of Science, 1-1-1 Ikenohata, Daito-ku, Tokyo 110-0008, Japan*
[c]*Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*
[d]*Department of Materials Sciences, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*
[e]*Creative Research Initiative "Sousei", Hokkaido University, Sapporo, Hokkaido 001-0021, Japan*

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Abstract

We developed a new biomaterial effective for nerve regeneration consisting of molecularly aligned chitosan with laminin peptides bonded covalently. Molecularly aligned chitosan was prepared from crab (*Macrocheira kaempferi*) tendons by ethanol treatment and 4 wt%-NaOH aqueous solutions to remove proteins and calcium phosphate, followed by deacetyl treatment using a 50 wt%-NaOH aqueous solution at 100 °C. Molecularly aligned tendon chitosan was chemically thiolated by reacting 4-thiobutyrolactone with the chitosan amino group. The introduction of thiol groups and their distribution to tendon chitosan and chitosan cast film were confirmed using ATR FT-IR, ^{1}H-NMR, and EDS. The 1.24 μmol/g of thiol groups introduced on the surface of tendon chitosan and the chitosan cast film was confirmed using ultraviolet (UV) spectra. Thiol groups of cysteine located at the end of synthetic laminin peptides were then reacted chemically with thiolated chitosan to form chitosan-S-S-laminin peptide. YIGSR estimated at 0.92 μmol/g and IKVAV estimated at 0.28 μmol/g on thiolated tendon chitosan were confirmed using UV spectra. YIGSR was estimated at 0.85 μmol/g and IKVAV was estimated at 0.34 μmol/g on the thiolated chitosan cast film.

Keywords: Crab tendon; Chitosan; 4-thiobutyrolactone; YIGSR; IKVAV; Immobilization

1. Introduction

The shells of crustaceans consist of chitin, calcium carbonate, and proteins [1]. Chitosan is generally prepared by *N*-deacetylating chitin from such shells. The chitosan thus obtained features such useful biological properties as biocompatibility, biodegradation, wound healing, and antibacterial action [2–6], thus attracting much attention for applications in chitosan-based biomedical materials such as drug delivery systems and wound-healing agents. Such biomedical properties of chitosan must be improved, however, for critical use in the adsorption of drugs and proteins and the adhesion of cells to biomedical materials, for example.

The advantages of chemically modified, improved chitosan include anti-HIV-1 activity [7], anticoagulant activity [8], drug transport such as with indometacin, and growth factors [9,10], and the transport of plasmid DNA [11,12]. Chemically modified chitosan is also used as scaffolds, thanks to its ability to adsorb cell-adhesive molecules [13–15] such as collagen, fibronectin,

*Corresponding author. Biomaterials Research Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan. Tel.: +81-29-851-3354X8765; fax: +81-29-854-7037.
E-mail addresses: matsuda.atsushi@nims.go.jp (A. Matsuda), kobayashi.hisatoshi@nims.go.jp (H. Kobayashi).

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vitronectin, and laminin. Kam et al. [16] and Tong et al. [17] reported that neural cell-adhesive molecules are adsorbed on the surface of substrates to effectively grow neural cells. Cell-adhesive molecules on the substrate easily desorb into the medium while cells are cultured.

We developed a new biomaterial for nerve regeneration consisting of molecularly aligned chitosan with laminin peptides bonded covalently. *Macrocheira kaempferi* was selected for its large tendons and their high mechanical strength for orthopedic use [18,19]. Its chitosan molecules are well aligned longitudinally. Tendon chitosan was reacted with 4-thiobutyrolactone to form thiolated tendon chitosan, which was then reacted with laminin peptides by disulfide bonding. The advantage of this reaction is that it maintains the structure of tendon chitosan and the biological activity of intact laminin peptides. Thiol groups and laminin peptides were introduced on tendon chitosan and analyzed qualitatively using infrared reflectance (FT-IR) spectra, ^{1}H-NMR spectra, polarized optical microscopy, and energy-dispersive X-ray spectroscopy (EDS). Amounts were determined quantitatively using UV spectra.

2. Materials and methods

2.1. Materials

Chitosan was kindly provided by Koyo Chemical Co., Japan. 4-thiobutyrolactone, imidazole, and acetic acid were obtained from Aldrich Co., USA and used without further purification. 5,5′-dithio-bis(2-nitrobenzoic acid) for quantitatively analyzing thiol groups was purchased from Aldrich Co., USA. The BCA protein assay kit for determining the amount of laminin peptides was obtained from Pierce Co., USA. Laminin peptides YIGSR and IKVAV were purchased from Bachem Co., Switzerland.

2.2. Preparation of chitosan samples

Chitosan cast film was prepared from commercially available chitosan powder as follows: Initially, 0.6 g of powder was dissolved in 20 ml of 3.0 wt% acetic acid aqueous solution, and the solution was cast on a laboratory dish. After drying, the resultant chitosan cast film was immersed in a 1 N-NaOH aqueous solution for 1 h to neutralize acetic acid, then washed in water 5 times to remove excess NaOH. Chitosan cast film was once again dried in a vacuum oven at room temperature.

Tendons 15–20 cm long were taken from crabs (*Macrocheira kaempferi*) to prepare chitin as reported elsewhere [18]. Crab tendons were washed with distilled water and treated in a 4 wt% NaOH aqueous solution at 100 °C for 8 h. Samples were then treated in ethanol of 95 wt% at 95 °C for 8 h. These treatments eliminated impurities in crab tendons such as protein and calcium phosphate. Samples were deacetylated with 50 wt% NaOH aqueous solution at 100 °C for 8 h in a nitrogen atmosphere 3 times to ensure complete deacetylation. Obtained samples were rinsed repeatedly with distilled water to remove excess NaOH. Final samples are referred as tendon chitosan.

2.3. Modification of chitosan with 4-thiobutyrolactone

Chitosan samples were thiolated using 4-thiobutyrolactone as described elsewhere [20]. Briefly, 2 g of tendon chitosan or chitosan cast film was immersed in 50 ml of water in a 100 ml 3-necked flask. An aqueous solution of imidazole (0.34 g in 2.5 ml) was added dropwise at room temperature in a nitrogen atmosphere, followed by the dropwise addition of 4-thiobutyrolactone (2.5 ml). After 5–200 h, the resulting reaction mixture was washed with 1 wt% sodium dodecylsulfate (SDS) and a 1 N-NaCl aqueous solution to remove adsorbing reagents by hydrophobic and electrostatic interaction, etc., and finally with distilled water using an ultrasonic cleaner, each for 10 min. After the removal of unreacted reagents and imidazole, thiolated tendon chitosan and thiolated chitosan cast film were dried in a vacuum.

2.4. Immobilization of laminin peptides on thiolated tendon chitosan and chitosan cast film

Thiolated tendon chitosan and chitosan cast film were immersed in 2 ml of 0.065 and 0.26 μmol/ml YIGSR and IKVAV PBS solutions (pH = 7.4) at room temperature for 24 h in an oxygen atmosphere. Obtained samples were washed with a 1% SDS aqueous solution and a 1 N-NaCl aqueous solution to remove adsorbing laminin peptide, then distilled water using an ultrasonic cleaner, each for 10 min.

2.5. Quantification of SH groups on thiolated tendon chitosan and chitosan cast film

The amount of thiol groups immobilized on tendon chitosan and chitosan cast film were determined by Ellman's method [21] as follows: An SH exchange reaction was conducted for 30 min. To measure the amount of thiol groups on the surface of the sample, the sample was used in its film state. The total amount of thiol groups on the sample was defined as that on the surface and that inside the sample was measured using the powdered sample, since the molecular size of Ellman's reagent was large and difficult to diffuse internal of the sample. The sample was immersed in PBS buffer (pH = 8.0) for 1 h, then an aqueous solution of 0.02 M 5,5′-dithiobis(2-nitrobenzoic acid) was added

to the PBS buffer containing thiolated samples and the mixture agitated for 30 min to complete the thiol exchange reaction. The absorbance of solutions was measured by the absorption of yellow coloration ($\lambda_{max} = 412$ nm; $\varepsilon_{max} = 1.3 \times 10^4$) using a multiplate reader (GENios, Tecan Japan Co. Ltd., Japan).

2.6. Determination of amounts of immobilized laminin peptides on thiolated tendon chitosan and chitosan cast film

Resultant samples were immersed in 2 ml of 2.5 mM dithiothreitor (DTT) PBS buffer solution to completely isolate laminin peptides, then the external solution was analyzed quantitatively using a BCA protein assay kit to measure absorbency (562 nm) using a multiplate reader (GENios, Tecan Japan Co. Ltd., Japan).

2.7. Characterization of laminin peptide modified tendon chitosan and chitosan cast film

ATR FT-IR spectra (FT-IR Spectrometer Spectrum 2000, Perkin Elmer, USA) were recorded at 4000–500 cm^{-1} using a Ga plate in a nitrogen atmosphere. Proton nuclear magnetic resonance (^{1}H-NMR) spectra (Spectrospin 300 Bruker BioSpin K. K., Switzerland) were measured after thiolated and intact chitosan samples were dissolved in a 3 wt% deuterated acetic acid deuterium oxide solution. The molecular alignment of samples was observed with a polarized optical microscope (BX 51 Olympus Co. Ltd., Japan) with crossed nicols by rotating the sample stage. The distribution of sulfur atoms in intact and thiolated tendon chitosan and chitosan cast film was analyzed using energy-dispersive X-ray spectroscopy (EDS) (SEM, Model EDSEM, JEOL, Tokyo, Japan).

3. Results and discussion

3.1. Thiolation of tendon chitosan and chitosan cast film

4-thiobutyrolactone was reacted chemically with tendon chitosan and chitosan cast film. Considering kinetic studies already reported [22,23], thiolactone first formed a reactive intermediate, HS–CH_2–CH_2–CH_2–C(O)–Im (where Im = 1-imidazolyl group), which reacted subsequently with nucleophilic functional groups of chitosan. Although thiolation sites and their distribution of chitosan molecules were not specified in the present study, an amino group was most likely the site of modification because these chemical groups are known to be reactive to electrophilic reagents [24,25] (Fig. 1).

ATR FT-IR of intact and thiolated tendon chitosan was measured to confirm the coupling reaction of 4-thiobutyrolactone with chitosan (Fig. 2). In original tendon chitosan, 1 absorption band was observed at 1595 cm^{-1} that corresponded to the peak of an amino group. After tendon chitosan was thiolated at reaction times of 25 and 96 h, 2 absorption bands observed at 1665 and 1543 cm^{-1} were assigned to amide I (C=O) and amide II (–NH) groups. The result of ATR FT-IR

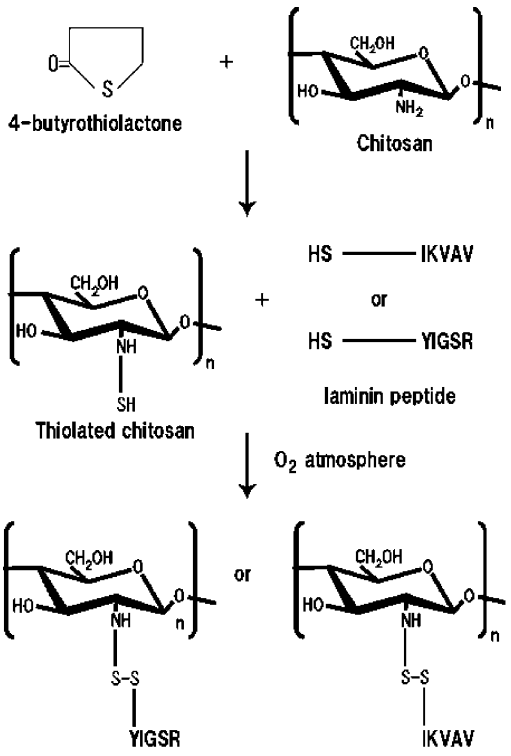

Fig. 1. Synthesized thiolated chitosan and laminin peptide-modified chitosan.

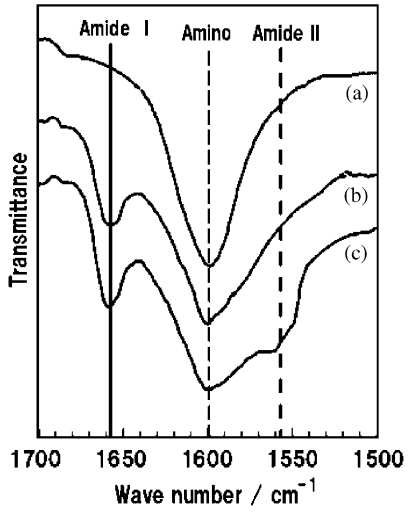

Fig. 2. FT-IR spectra of (a) intact tendon chitosan, (b) thiolated tendon chitosan in a reaction of 25 h, and (c) thiolated tendon chitosan in a reaction of 96 h.

spectra of thiolated chitosan cast film was similar to that of thiolated tendon chitosan, indicating that amino groups of chitosan and 4-thiobutyrolactone reacted to form amide bonds. Thiol groups were not detected by FT-IR, however, since the peak intensity was very weak. To detect thiol groups on thiolated tendon chitosan, ^{1}H-NMR of thiolated tendon chitosan was measured (Fig. 3). After tendon chitosan was thiolaed, a new peak appeared at 2.1 ppm corresponding to the proton of the thiol group. The result of ^{1}H -NMR spectra of thiolated chitosan cast film was similar to that of thiolated tendon chitosan. Results of ATR FT-IR and ^{1}H -NMR indicate that thiol groups on tendon chitosan and chitosan cast film are introduced through covalent bonding, and introduced thiol groups on tendon chitosan and chitosan cast film do not react inter- or intramolecularly, i.e., introduced thiol groups react with other materials having thiol groups. To determine the distribution of thiol groups on tendon chitosan and chitosan cast film, we analyzed sulfur atoms in the cross-section of these samples using EDS (Fig. 4). Figs. 4a and b show SEM images and the mapping of sulfur atoms for intact and thiolated tendon chitosan. Figs. 4c and d show images for intact and thiolated chitosan cast film. In results for Figs. 4b and d, sulfur atoms are distributed over the whole area of thiolated chitosan samples. No sulfur atoms were detected, however, in intact chitosan samples. These results indicate that the reactant was diffused and reacted in tendon chitosan and chitosan cast film, since these chitosan samples were swollen in water. The water content of tendon chitosan was 40.1 wt% and that of chitosan cast film 37.5 wt%.

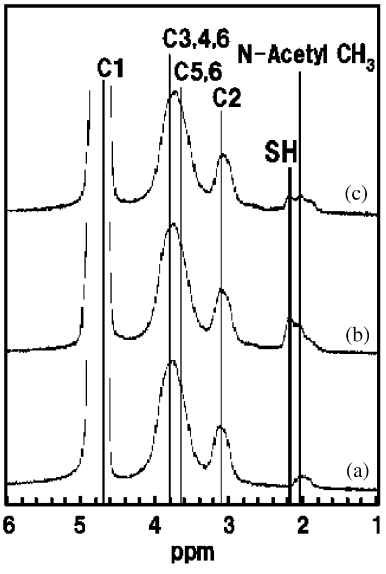

Fig. 3. ^{1}H-NMR spectra of (a) intact tendon chitosan, (b) thiolated tendon chitosanon in a reaction of 25 h, and (c) thiolated tendon chitosan in a reaction of 96 h.

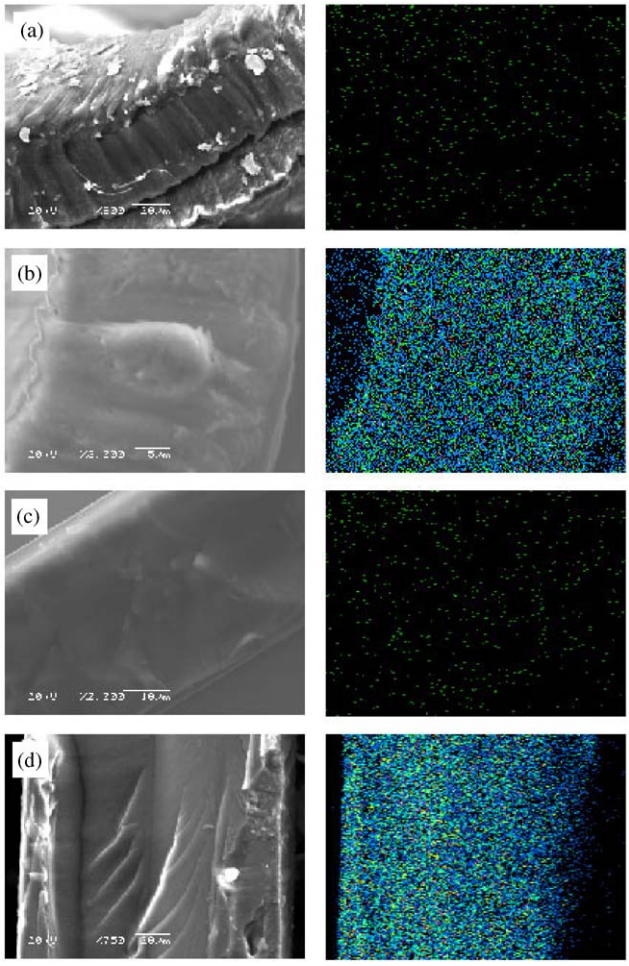

Fig. 4. EDS of (a) intact tendon chitosan, (b) thiolated tendon chitosan, (c) intact chitosan cast film, and (d) thiolated chitosan cast film. Images at left are SEM and those at right EDS images of sulfur atoms.

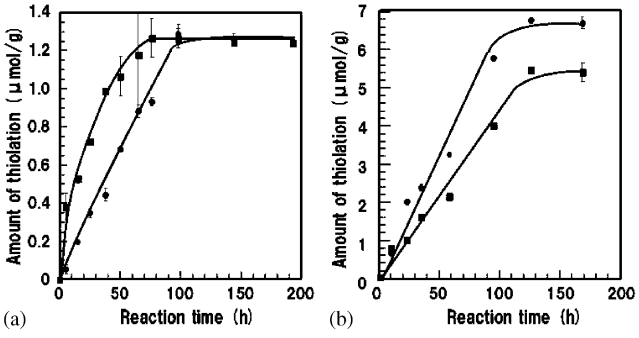

Fig. 5. Degrees of thiolation (a) on the surface, (b) overall for tendon chitosan (●) and chitosan cast film (○).

We evaluated the influence of reaction time on the introduction of thiol groups on the surface and overall tendon chitosan and chitosan cast film, calculating the amount of introduced thiol groups on the surface (Fig. 5a) and overall against the weight of chitosan samples

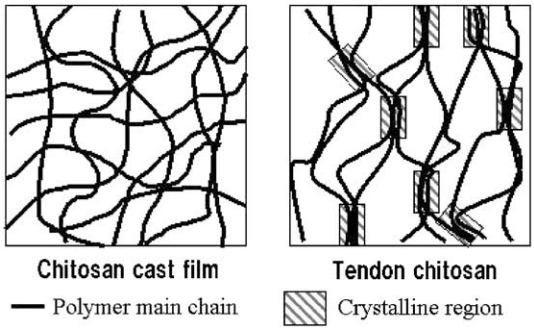

Fig. 6. Structure of tendon chitosan and chitosan cast film.

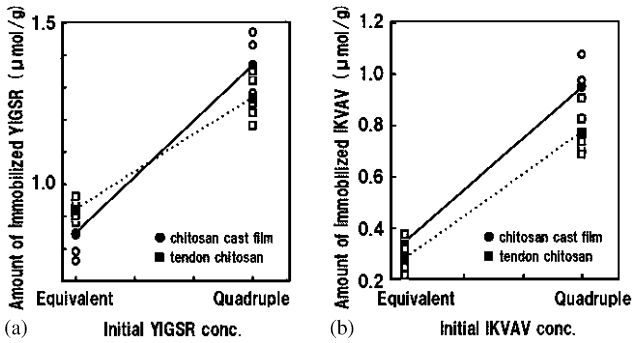

Fig. 7. Amount of (a) modified-YIGSR and (b) modified-IKVAV on tendon chitosan (squares) and chitosan cast film (circles). Closed symbols denote averages and open symbols measurement data ($n = 5$).

(Fig. 5b). The amount increased with increasing reaction time. The initial reaction of 4-thiobutyrolactone on the surface of tendon chitosan was faster than that of chitosan cast film by up to 80 h, since the total surface area of tendon chitosan was greater than that of chitosan cast film. The amount of introduced thiol groups on the surface of tendon chitosan and chitosan cast film saturated at over 90 h and 1.24 μmol/g at 193 h. The initial reaction of 4-thiobutyrolactone to total tendon chitosan was slower than that of chitosan cast film at up to 100 h. The amount of thiol groups introduced on tendon chitosan was lower than that on chitosan cast film. Based on the results of X-ray diffraction (XRD) for swollen tendon chitosan and chitosan cast film (data not shown.), tendon chitosan had an organized structure, but chitosan cast film was amorphous. The results of water content and XRD indicated that the reactant was diffused and reacted with amino groups in internal tendon chitosan and chitosan cast film, but did not react with amino groups containing crystalline tendon chitosan (Fig. 6).

3.2. Immobilization of laminin peptides on tendon chitosan and chitosan cast film

Laminin peptide immobilization generally requires that matrices be premodified. To achieve this activation, we introduced sulfhydryl groups onto tendon chitosan and chitosan cast film to get a reaction between sulfhydryl groups of thiolated chitosan and laminin peptide (Fig. 1). The amount of covalently bonded laminin peptides on thiolated tendon chitosan and thiolated chitosan cast film is shown in Figs. 7a and b. When purification was made with laminin peptide adsorbed chitosan samples as controls, only negligible amounts of laminin peptides were detectable. We also confirmed that decreasing amounts of laminin peptide in the external solution after this reaction and isolated amounts of laminin peptide from the sample are equal. These results indicate that thiol groups of laminin peptides reacted chemically with thiolated chitosan samples. When the number of laminin peptides was equivalent (0.065 μmol/ml) to thiol groups on thiolated chitosan samples, the amount of reacted laminin peptide was 22–74% compared to introduced thiol groups. The amount of modified laminin peptide differed little between thiolated chitosan samples ($p > 0.05$), but the amount of modified YIGSR was higher than that of modified IKVAV on both thiolated chitosan samples, i.e., the reactivity of YIGSR is higher than that of IKVAV. We consider the number of thiol groups per volume for laminin peptide to depend on this reaction. If this value is high, thiol groups of laminin peptides can attack thiol groups on thiolated chitosan samples more easily. This value for YIGSR is higher than that of IKVAV, since both laminin peptides have 1 thiol group per molecule and the molecular weight of YIGSR (MW = 966.1) is lower than that of IKVAV (MW = 2017.4). When a quadruple number of laminin peptides was reacted on thiolated chitosan samples, the ratio of reacted YIGSR and IKVAV was from 100% to 65%, indicating that this reaction proceeds stoichiometrically.

The coupling reaction of sulfhydryl groups advances easily at higher pH [26], i.e., decreasing H^+ concentration in rising pH leads, in turn, to larger amounts of negative thiolate anions, S^-, representing the active form for oxidation. According to the literature [27], the p*K*a of thioglycolic acid shifts lower to react with amino groups of chitosan. In this study, we postulate that the p*K*a of 4-thiobutyrolactone shifts lower to react with the amino group on chitosan samples reinforcing oxidation.

When the optical microscopic image of tendon chitosan (Fig. 8a) was rotated 45°, its coloration disappeared completely (Fig. 8b), indicating chitosan molecules are aligned well along the longitudinal axis. Figs. 8c and e show polarized optical microscopic images of YIGSR- and IKVAV-modified tendon chitosan. Figs. 8d and f show their images at the 45°-rotated position, also demonstrating coloration change. These results indicate that the molecularly aligned structure of tendon chitosan is not changed by

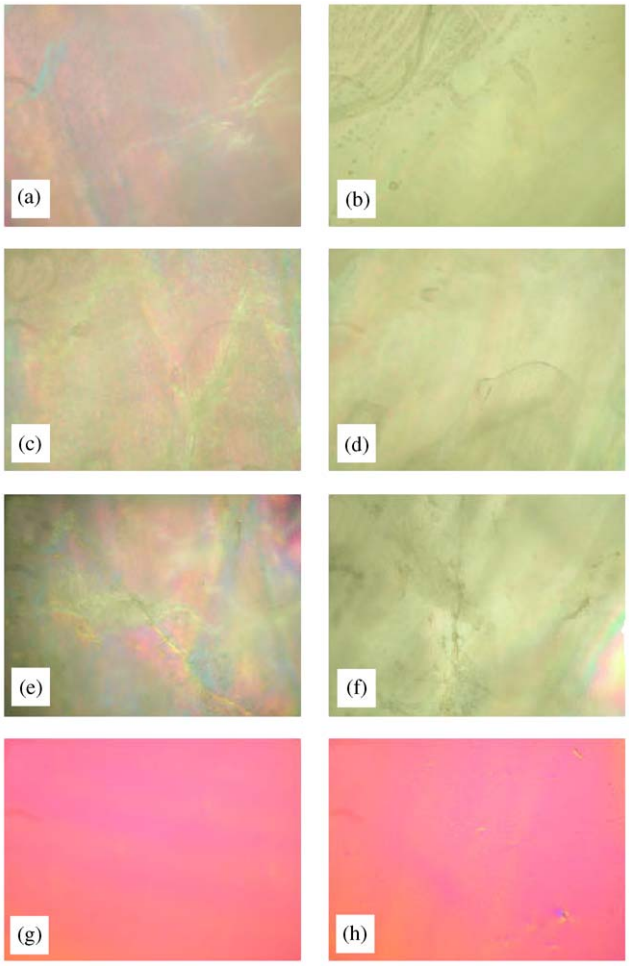

Fig. 8. Polarized optical microscopic images observed under crossed Nicols; (a) crab tendon, (b) rotated 45°, (c) YIGSR-modified crab tendon, (d) rotated 45°, (e) IKVAV-modified crab tendon, (f) rotated 45°, (g) chitosan cast film, and (h) rotated 45°.

thiolation or laminin peptide modification. Figs. 8g and h show the polarized optical microscopic images of chitosan cast film and its image rotated 45°. Coloration does not change because the film is amorphous.

We conclude from these results that the immobilization of laminin peptides on tendon chitosan and chitosan cast film under mild reaction conditions is successful while maintaining the original molecular structure of tendon chitosan intact.

4. Conclusions

We immobilized laminin peptides on crab tendon chitosan and chitosan cast film by covalent bonding under mild oxidation while maintaining the molecular structure of tendon chitosan intact. YIGSR and IKVAV were used as a sample biomolecule for modification of chitosan samples. This 2-step modification on tendon chitosan and chitosan cast film is widely applicable to conjugation with biomolecules, such as proteins or oligopeptides [28], to improve the functionality of chitosan samples. We are currently studying the immobilization of physiologically active substances on chitosan matrices to develop new biomaterials; e.g., nerve regeneration guide tubes and artificial scaffolds.

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