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Spectroscopic study on the conformation of serum albumin in film state

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Protein is a promising material for fabricating the biocompatible films used in the biomedical fields and food industry. Previously, we successfully prepared a water-insoluble albumin film possessing native albumin properties such as resistance to cell adhesion and drug-binding ability. Here, I quantitatively investigated the conformation of albumin in a film state using circular dichroism (CD) spectroscopy. The albumin film was prepared by crosslinking albumin with ethylene glycol diglycidyl ether (EGDE). CD measurements of albumin films revealed that approximately 70% of the α-helical structure was retained after film formation. Albumin molecules in the films acquired high stability. The conformation of albumin was completely retained even after heating at 100 °C for 1 h. For comparison, crosslinked albumin film was also prepared using glutaraldehyde (GA). Unlike EGDE-crosslinking, GA-crosslinking induced significant conformational changes in albumin; 46% of the α-helical structure was destroyed in GA-crosslinked albumin films. Cell adhesion studies showed that EGDE-crosslinked albumin film maintained the cell-nonadhesive property inherent in native albumin. This property was lost in GA-crosslinked albumin film, and cells adhesion occurred at a level comparable to that of cell culture dishes. These results indicate that EGDE-crosslinking is a useful method for preparing albumin films in which the native albumin structure and property are retained. The approach described here provides valuable information for creating protein films possessing high functionality.

[**Key words:** Serum albumin; Protein film; Crosslinking; Circular dichroism spectropolarimetry; Epoxy compound]

Extensive effort has been put into the preparation of films using proteins because of their superior biocompatibility and biodegradability and their similarities to the extracellular matrix. Some protein films were created using several types of proteins such as collagen, gelatin, elastin, keratin, silk fibroin, soy protein, zein, and casein (1–7). Such films are utilized in tissue engineering applications, wound dressing, and food packing. However, because proteins are highly sensitive to external stress, native structures and functions of proteins are lost in these conventional protein films during the process of film fabrication, which includes physical/chemical treatments and a drying step. Proteins possess diverse functionalities, evidenced by the roles they play in living systems; these functionalities include catalysis, transport, recognition, and communication. Conventional films, fabricated primarily to harness the biocompatible and biodegradable properties of proteins, do not sufficiently utilize the capabilities of proteins. Indeed, only several proteins have been researched for the development of protein films. Proteins possessing specific functions, such as enzymes, antibodies, cytokines, and transport proteins, remain largely uninvestigated in this area of research. Thus, the next challenge in the film preparation technology using proteins is the creation of the protein films having high functionality through the development of the methods to prepare the films without losing the structures and functions of native protein as well as the usage of the various kinds of proteins with specific functions.

To create a protein film that retains the functional properties of its component protein, we focused on serum albumin. Albumin can prevent adsorption of other proteins and cell adhesion on its coated surface, and has the ability to bind many compounds such as drugs, organic dyes, and short-chain fatty acids (8–11). Previously, we successfully prepared a water-insoluble free-standing albumin film in which the native albumin properties, such as drug-binding ability and resistance to protein adsorption and cell adhesion, were retained. This water-insoluble free-standing albumin film was prepared by crosslinking albumin with ethylene glycol diglycidyl ether (EGDE) and then casting the crosslinked albumin solution (12,13). Because the functional properties of proteins depend on their three-dimensional conformation, the fact that the albumin in the film state maintained its native properties indicates that the structure of albumin was retained. However, the details of albumin structure in a film state remain unclear. In the present study, I quantitatively investigated the conformation of albumin molecules in a film prepared by crosslinking with EGDE, using circular dichroism (CD) spectropolarimetry. Albumin structure in the film crosslinked with EGDE was also compared with that in the film crosslinked with glutaraldehyde (GA), which is widely used for crosslinking proteins (14,15). Cell adhesion on these albumin films was also examined to assess the influence of albumin structure on the behavior of cell adhesion. These results will provide useful information for preparing protein films in which the structures and functions of native proteins are retained.

MATERIALS AND METHODS

Crosslinking of albumin with EGDE Crosslinking of albumin with EGDE was conducted as described previously (Fig. 1) (12,16). Bovine serum albumin (BSA;

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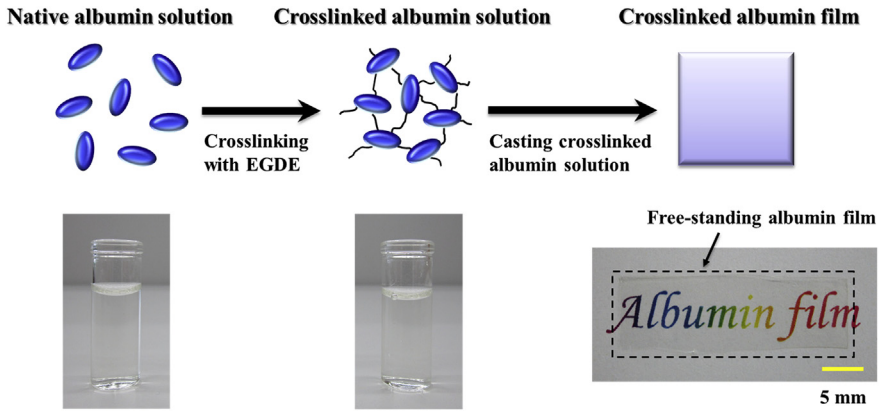

FIG. 1. Schematic of the process for preparing a crosslinked albumin film using EGDE; images were acquired at each step.

Sigma, MO, USA) was dissolved in phosphate-buffered saline (PBS, pH 7.4) to yield a 3% solution, which was then reacted with 215 mM EGDE (Wako, Osaka, Japan) with vigorous stirring for 24 h at 25 °C. The reaction mixture was dialyzed for 3 days at room temperature against Milli-Q water using cellulose tubing (molecular weight cutoff = 12 kDa; Nihon Medical Science, Gunma, Japan) to remove the unreacted EGDE. The reaction mixture was then adjusted to a final concentration of 2% by adding Milli-Q water, sterilized by filtering through a 0.22-μm filter, and stored at 4 °C.

Determination of conformational changes in albumin molecules CD spectropolarimetry was used to determine the conformations of native albumin and crosslinked albumin in solution, as well as those in crosslinked albumin films. CD measurements were performed using a J-820 spectrophotometer (JASCO Co. Ltd., Tokyo, Japan) over the range of 190–250 nm. Samples of albumin solution before and after crosslinking with EGDE were dissolved in Milli-Q water at a concentration of 10 μg/ml; measurements were performed using a 1-cm path length quartz cuvette. Each spectrum was corrected for baseline by subtracting the spectral contributions of the solvent and the quartz cuvette. The results are expressed as mean residue ellipticity (deg cm^2/dmol) using the following equation (17):

$$[\theta] = (\theta \times M_0)/(10000 \times C \times l) \quad (1)$$

where θ is the observed ellipticity (mdeg), l is the optical path length (cm), C is the protein concentration (g/ml), and M_0 is the mean residue molecular weight (=113) calculated using the molecular weight of albumin (=66,000) and the number of amino acid residues in albumin (= 582) (18).

Structural analysis of albumin film samples was performed in accordance with the method described by Sivaraman et al. (17). Film-coated quartz plates for CD measurements of albumin-film samples were prepared as follows. EGDE-crosslinked albumin solutions, or a mixture of BSA (2%) and glutaraldehyde (9 mM, Wako) in Milli-Q water, were poured onto a quartz plate (3 × 3 × 0.03 cm; Vidrex Co. Ltd., Fukuoka, Japan). After 30 min of incubation, the plates were placed on the rotor of a spin coater (1H-DX2; Mikasa Co. Ltd., Tokyo, Japan), rotated at 500 rpm for 5 s, and dried overnight at 37 °C for film formation. The resulting quartz plates, coated with EGDE- or GA-crosslinked albumin film, were placed in the CD spectrometer, where CD measurements of each film sample were made in an atmospheric environment. Each spectrum was corrected for baseline by subtracting the spectral contribution of the quartz plate. UV absorbance was measured after conducting CD measurements, using the same plates to determine the amount of albumin in the film (17,19). The UV absorbance of the film samples was measured at 220 nm using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) and ensuring that the light beam passed through the position where the CD beam had passed. The wavelength of 220 nm was selected because peptide bonds emit strong signals in the region of 180–230 nm, and a previous report suggested that absorbance at 220 nm remains constant regardless of conformational changes in proteins (19). The UV absorbance data were corrected by subtracting the absorbance contribution of the quartz plate. As stated by the Beer–Lambert Law, the observed absorbance, A, is expressed by

$$A_{220} = \varepsilon \times C \times l \quad (2)$$

where ε is the extinction coefficient of the protein, C is the protein concentration (g/ml), and l is the optical path length (cm). The term $C \times l$ has units of g/cm^2; assuming that absorbance is dependent only on the total mass of albumin per unit area which the light beam passes through, regardless of whether albumin is in solution or has formed a film, the term $C \times l$ corresponds to the amount of albumin in the film per unit area (17). By substituting the $C \times l$ term, calculated from Eq. 2, into Eq. 1, the CD data on albumin film samples were converted into values of mean residue ellipticity. ε was obtained from the slope of the calibration curve of the A_{220} - C graph, which was prepared using the dilution series of the albumin solution and 1-cm path length quartz cuvette.

For the thermal treatment of albumin samples, native albumin solution and EGDE-crosslinked albumin solution were incubated at 95 °C for 15 min, and EGDE-crosslinked albumin film was incubated at 100 °C for 1 h in an atmospheric environment. The α-helix content of each sample was determined from the ellipticity at 222 nm (20). The reduction in α-helix content after various treatments, such as crosslinking, film formation, or heating, was estimated using the following equation:

$$\text{Reduction in } \alpha\text{-helix content } (\%) = [(Na - Nc)/Na] \times 100 \quad (3)$$

where Na or Nc is the ellipticity of albumin samples before or after various treatments, respectively, at 222 nm.

Cell adhesion behavior on crosslinked albumin films EGDE-crosslinked albumin solutions, or a mixture solution of BSA (2%) and glutaraldehyde (9 mM) in Milli-Q water, were poured onto cell culture dishes and dried overnight at 37 °C to coat the dishes with EGDE- or GA-crosslinked albumin films. Then, the films were immersed in 100 mM glycine in PBS for 1 h at 37 °C and washed with PBS. The stromal cell line PA6, derived from mouse calvaria, was grown on culture dishes in Minimum Essential Medium Alpha Medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. PA6 cells were collected using a 0.25% trypsin/EDTA solution and resuspended in α-MEM-based culture medium. Then, the cells were plated on albumin film-coated dishes at a density of 2.5 × 10^4 cells/cm^2. An intact cell culture dish and native albumin-coated dish, prepared by

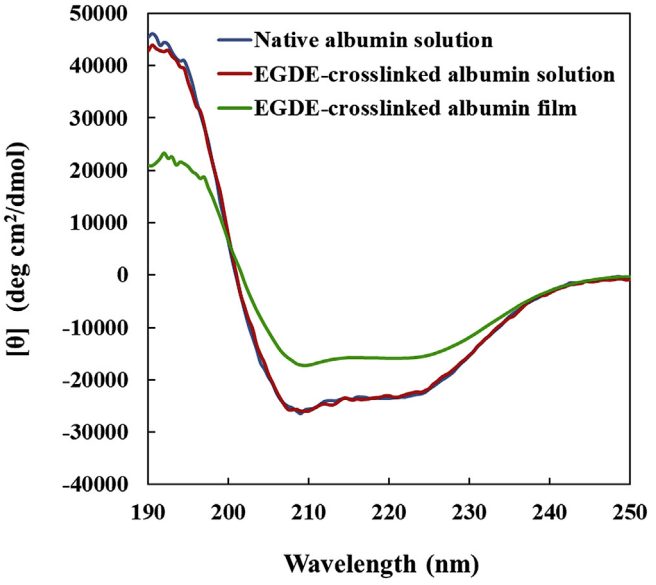

FIG. 2. CD spectra of a native albumin solution, EGDE-crosslinked albumin solution, and EGDE-crosslinked albumin film.

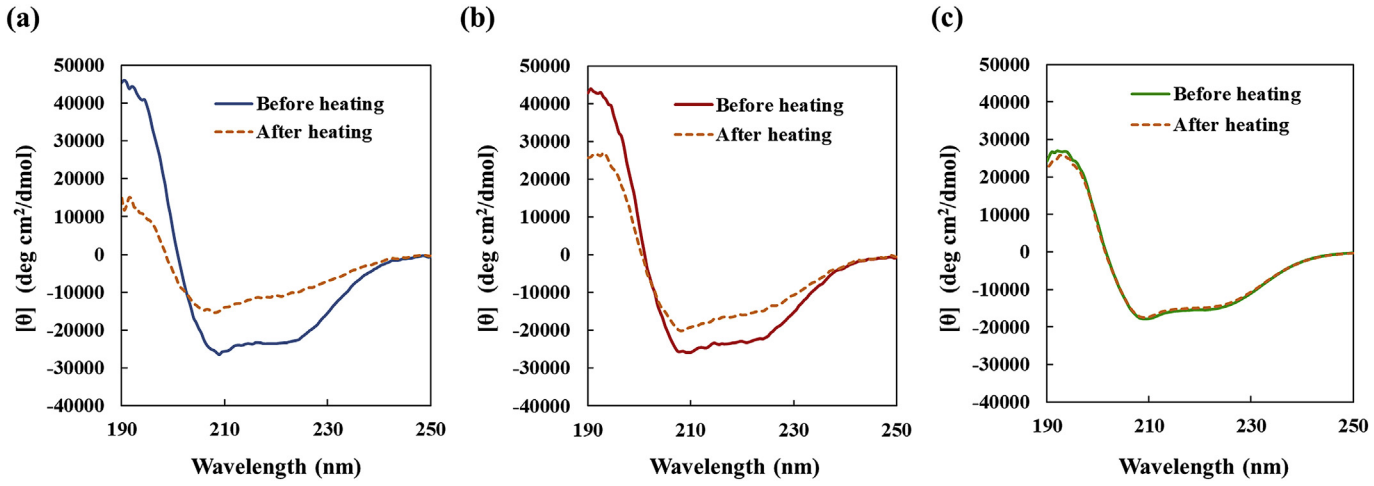

FIG. 3. Conformational changes in albumin molecules upon heating. (a) CD spectra of native albumin solution before and after heating at 95 °C for 15 min. (b) CD spectra of EGDE-crosslinked albumin solution before and after heating at 95 °C for 15 min. (c) CD spectra of EGDE-crosslinked albumin film before and after heating at 100 °C for 1 h.

adding a 3% BSA solution in PBS to cell culture dishes, were used as controls. After a 5-h incubation in a humidified 5% CO_2 incubator at 37 °C, the cells were washed with PBS three times to remove unattached cells. Attached cells were collected by trypsinization, and the cell number was counted. Cell counting and cell observations were performed using a hemocytometer and a phase contrast microscope (IX70; Olympus, Tokyo, Japan), respectively.

RESULTS AND DISCUSSION

Preparation of water-insoluble crosslinked albumin films using EGDE and GA Serum albumin is a water-soluble protein. An albumin film prepared from native albumin easily dissolves in water. Therefore, it is necessary to crosslink albumin in order to render an albumin film water-insoluble. However, excess crosslinking can disrupt the structure of albumin, resulting in a loss of native albumin properties. To suppress this excess crosslinking of albumin, we optimized the crosslinking method in our previous studies (12). In those studies, we prepared a stock solution of crosslinked albumin by reacting albumin with EGDE of the lowest concentration necessary to obtain a water-insoluble film. We then removed the unreacted EGDE by dialysis, thus minimizing the amount of EGDE involved in crosslinking (Fig. 1). No difference in the solution of albumin was observed between before and after crosslinking. There was no precipitation of albumin after the crosslinking reaction with EGDE. The water-insoluble albumin film was prepared by casting the crosslinked albumin solution. The resultant albumin film was colorless and transparent.

On the other hand, precipitation of albumin occurred when albumin was crosslinked with GA. This precipitation was observed even though the concentration of GA was reduced to such a low level that a water-insoluble film was no longer obtainable due to insufficient crosslinking. Therefore, I prepared the GA-crosslinked film by casting the mixture solution of albumin and GA; this was performed without preparing the stock solution of crosslinked albumin, as had been done previously, when using EGDE crosslinking. The concentration of GA was experientially determined, based on the pilot study, using the mixture solution of albumin and dilution series of GA. The lowest GA concentration (9 mM) necessary for preparing the water-insoluble film was used in this study to prevent the excessive crosslinking of albumin as much as possible.

Conformational changes in albumin molecules during the process of film preparation using EGDE To investigate conformational changes in albumin molecules after crosslinking with EGDE or film formation, CD measurements were performed in the range of 190–250 nm (Fig. 2). In agreement with previous reports, the CD spectrum of native albumin showed characteristic minima at 208 and 222 nm, which corresponded to an α-helical structure (20,21). BSA is known to be composed mostly of α-helixes; one study estimates the secondary structural components of BSA to be 68% α-helix and 18% β-structure (22). No marked difference in CD spectra was observed between the albumin samples before and after crosslinking. This indicates that no significant structural changes occurred in albumin after crosslinking with EGDE. Film formation induced a slight decrease in CD signals; 29 ± 2% of the α-helical structure in albumin molecules was disrupted upon film formation. Thus, approximately 70% of the α-helical structure was retained after the process of film formation, including the crosslinking reaction and the drying step.

Resistance of EGDE-crosslinked albumin to heating-induced denaturation Conformational changes in albumin samples after heating were examined using CD measurements (Fig. 3). When native albumin solution was heated at 95 °C for 15 min, CD

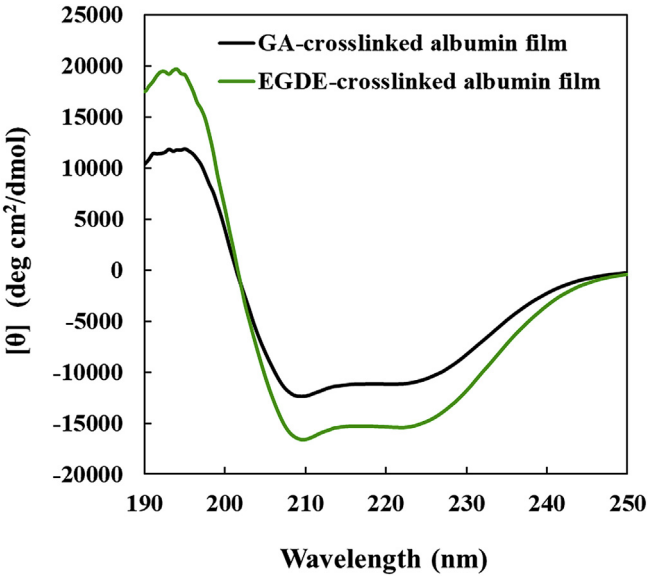

FIG. 4. CD spectra of EGDE- and GA-crosslinked albumin films.

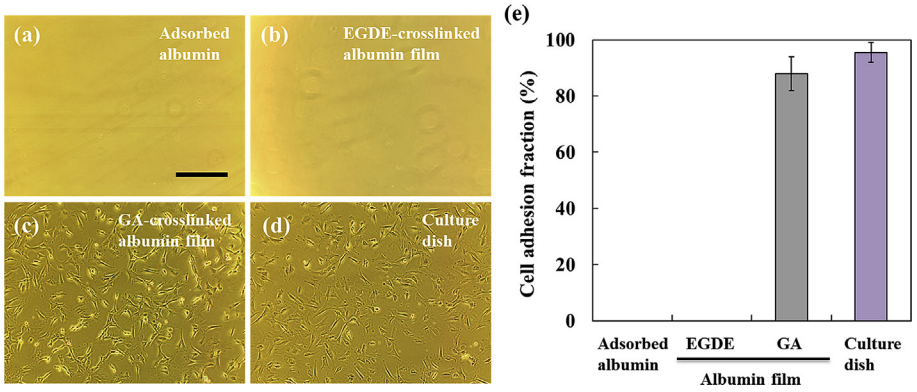

FIG. 5. Cell adhesion on various albumin surfaces. Phase-contrast micrographs of PA6 cells cultured on (a) native albumin-coated dish, (b) EGDE-crosslinked albumin film, (c) GA-crosslinked albumin film, and (d) cell-culture dish. Scale bar: 300 μm. (e) Fraction of adherent PA6 cells on various albumin surfaces. Data are represented by the mean ± SD (n = 4).

signals around 208 and 222 nm were significantly decreased, and together with the minimum at 208 nm tended to shift to a lower wavelength, which suggests that heating induced substantial conformational changes, such as loss of α-helixes and increased random-coil conformation, in the albumin molecules (Fig. 3a). 57 ± 3% of the α-helical structure was destroyed by heating. The extensive conformational changes, caused by heating, were suppressed by the crosslinking of albumin. This was evidenced by a lower rate of reduction in the α-helix content (34 ± 2%) of the crosslinked albumin solution, which was heated under the same conditions (Fig. 3b). A crosslinked network formed in the albumin molecules, at the intermolecular and intramolecular levels, may contribute to the prevention of heating-induced conformational changes. EGDE primarily reacts with lysine and histidine residues in albumin; the reaction efficiency of EGDE is estimated to be 28% (12). Interestingly, the albumin in film state was completely resistant to heating-induced conformational changes even when heated at 100 °C for 1 h; this shows the high stability of albumin molecules in the film (Fig. 3c). I hypothesize that the molecules of EGDE, which react with albumin at one epoxy group and retain another epoxy group intact, remain in the crosslinked albumin solution. Chemical bonds are formed by the intact epoxy groups, and physical interactions are generated among the albumin molecules during the process of film formation. In this process, the albumin molecules move closer to one another as the solvent of crosslinked albumin solution is evaporated. These chemical and physical interactions, formed among the albumin molecules, impart albumin with high stability in the film state.

Comparison of EGDE-crosslinked albumin film with GA-crosslinked albumin film I compared the α-helical structure of albumin in film crosslinked with EGDE with that in film crosslinked with GA. The CD spectra of EGDE- and GA-crosslinked albumin films are shown in Fig. 4. Large-scale conformational changes in albumin were observed in GA-crosslinked albumin films; the α-helix content of GA-crosslinked film was 24 ± 5% less than that of EGDE-crosslinked film. This corresponds to the loss of 46% in α-helical structure compared with that of native albumin. These results indicate that EGDE crosslinking is superior to GA crosslinking for the maintenance of native albumin structure. Epoxy compounds generate hydroxyl groups after a crosslinking reaction, enhancing the hydrophilicity of crosslinked materials (23,24). Although the detailed molecular mechanism is unclear, this increased hydrophilicity may help prevent conformational changes and aggregation of albumin.

Cell adhesion on EGDE- and GA-crosslinked albumin films Cells on native albumin-coated surfaces show no adhesion; this is a well-known characteristic of albumin. To assess the cell-adhesive property of crosslinked albumin films, PA6 cells were seeded on albumin films crosslinked with EGDE or GA. Representative photomicrographs, and a quantitative assessment of cell adhesion, on various albumin substrates after a 5-h incubation are shown in Fig. 5. The behavior of cell adhesion on each surface was evaluated after 5 h because additional cell adhesion did not occur on the surfaces even when cells were cultured for more than 5 h. The results show that cell adhesion did not occur on EGDE-crosslinked albumin film in which 71% of the α-helical structure was retained (Fig. 2); this is similar to the behavior observed on native albumin-coated dishes (Fig. 5a,b and e). Whereas, cells adhered well to GA-crosslinked albumin film in which 46% of the α-helical structure of albumin was destroyed (Fig. 4); the degree of this cell adhesion was comparable to that on cell-culture dishes (Fig. 5c,d and e). Thus, the cell-nonadhesive property of native albumin was maintained in EGDE-crosslinked albumin film but not in GA-crosslinked albumin film. These results indicate that the albumin structure in the film greatly affects the behavior of cell adhesion, and highlight the importance of selecting the appropriate crosslinker for creating an albumin film in which the native albumin structure and function are retained. It is important to note that cell adhesion occurred on the EGDE-crosslinked gelatin film (Fig. S1). Therefore, cell-nonadhesiveness observed in the EGDE-crosslinked albumin film was derived from the property of albumin, and was not due to the influence of EGDE.

Conclusions In this study, water-insoluble albumin films were prepared using the two crosslinkers, EGDE and GA, under optimized crosslinking conditions. In the film prepared using EGDE, 71% of the α-helical structure was retained, and the film maintained the cell-nonadhesive property inherent in native albumin. On the other hands, in the film prepared using GA, 46% of the α-helical structure was destroyed, and cell adhesion occurred at a level comparable to that observed on cell-culture dishes. These results indicate that EGDE crosslinking is a promising method for preparing an albumin film in which the structure and property of native albumin are retained. The approach described here provides useful information for the preparation of water-insoluble protein films with retained structural and functional properties, and the creation of novel protein films having high functionality.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.09.015.

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