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Preparation and Mechanical Evaluations of a Novel Keratin-Chitosan-Gelatin Composite Film

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Abstract: Keratin-chitosan composite film was prepared by casting the mixed solution of both biopolymers in 75% acetic acid. Although keratin film without any additive is very fragile, 10-30wt% of chitosan addition gave strong and flexible film (ultimate strength: 27-34 MPa, ultimate elongation: 4-9%). Glycerol (20 wt%) also afforded flexibility to keratin film (ultimate strength: 1MPa, ultimate elongation: 28%). Further addition of chitosan to glycerol-containing keratin film increased the ultimate strength to 9-14MPa but gave little effect on ultimate elongation. These data suggest that mechanical properties of keratin film are adjustable by appropriately adding chitosan and glycerol. Waterproof characteristics such as swelling behavior and mechanical properties after swelling were much ameliorated for the composite film compared with keratin-chitosan-gelatin films, respectively. Furthermore, keratin-chitosan composite film as well as chitosan film decreased bacteria number when the bacteria suspension was treated with a film owing to the irreversible adsorption of bacteria onto the film. The composite film as well as keratin-chitosan-gelatin films supported fibroblast attachment and proliferation, demonstrating to be a good substrate for mammalian cell culture. A series of chitosan/gelatin composite films were prepared by solvent evaporation and characterized for oxygen permeability, optical transmittance, water absorptivity and mechanical properties. Chitosan/gelatin composite films are more permeable, transparent, flexible and biocompatible films and could potently be used for contact lens material.

Keywords: Keratin · Chitosan · Composite film · Antibacterial activity · Cell substrate · Gelatin

INTRODUCTON

Chitin is the second most abundant natural biopolymer (cellulose being the most abundant). It is widely distributed throughout the nature as the exoskeleton material of crustaceans and insects.

Structurally, chitin is a naturally abundant mucopolysaccharide consisting of a 2-acetamido-2-deoxy--D-glucose repeat unit through a (1→4) linkage. The most easily exploited sources are the protective shells of crabs and shrimps [1].

Chitosan Increased vascularization and organization were noted for the chitosan films and gels. Evidence pointed to the migration of stromal cells and their differentiation into structurally needed cell types as the fundamental cause of decreased wound healing times and improved wound tissue organization [2]. Chitosan is a very interesting biomaterial since it is readily available and can be easily made into a variety of useful forms such as membranes, sponges, fibers and beads as well as powders and solutions [3].the polymer is called chitin when the degree of deacetylation is below 50% and chitosan when the degree of deacetylation is above 50% or above. Of the two polymers, chitosan is more often investigated for use both inside and outside of the body. Chitosan is used because it is soluble in dilute organic acids, allowing the hydroxyl and amino groups to be utilized in chemical reactions. Upon dissolution, the amino group in chitosan becomes protonated, resulting in a positively charged polysaccharide that can attract and promote cell adhesion. [4].

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It has a unique chemical structure as a linear polyelectrolyte with a high charge density as well as reactive hydroxyl and amino groups. Chitosan is an excellent flocculent, adhering to negatively charged surfaces with biocompatible, non-toxic, biodegradable and fungicidal activities; furthermore, it is possible to modify the chitosan as an antimicrobial polymer which makes it a very attractive biomaterial. Chitosan has shown to facilitate wound healing, reduce serum cholesterol levels and to stimulate the immune system [3].

Keratin is the major structural fibrous protein providing outer coverings such as hair, wool, feathers, nail and horns of mammals, reptiles and birds [5]. From the amino acid analysis, keratin is found to be characteristically abundant in cysteine residues (7-20% of the total amino acid residues) [6]. These cysteine residues are oxidized to give inter-and intra-molecular disulfide bond, which may result in the mechanically strong three-dimensionally linked network of keratin fiber. The flexible but tough property of hair and wool might be attributed to this characteristic structure of keratin fiber.

Although various attempts have been made to extract keratin from hair or wool, most of them gave keratin carrying chemically modified cysteine [7-10]. Extraction of unmodified reduced keratin solution has never been done because of its instability. Recently, we reported the preparation of a stable aqueous solution of reduced keratin [11]. Although a keratin film prepared by casting the keratin solution without any additive was too fragile to handle, addition of glycerol improved the property of a film to give a transparent, fairly strong, flexible and biodegradable film [11]. The mouse fibroblast cells could proliferate well on the keratin film [12], suggesting the biocompatibility of keratin. Thus, keratin is expected to be applicable for biomedical use in a similar manner to collagen and gelatin. Although the keratin film containing glycerol showed appropriate strength and flexibility as described above, glycerol dissolved out in the aqueous environment, resulting in the fragile film again.

Chitosan, which is composed of glucosamine and N-acetylglucosamine, is a non-toxic, biocompatible, biodegradable and hydrophilic polymer [13]. Its applications range from environmental technology, medicine, cosmetics, agriculture, food and biotechnology to chemistry [14]. Recently, research has been focused on its application in medicine due to its antibacterial and biocompatibility. Chitosan can be used to make blood vessels, surgical sutures and wound healing agents. The ability of chitosan to form films permits its use in medicine as artificial skin and contact lenses [15]. Mao *et al.*, studied the properties of chitosan/gelatin membrane modified by hyaluronic acid and the chitosan/gelatin membrane showed good biocompatibility [16].

MATERIALS AND METHODS

Preparation of Keratin Solution: Keratins were extracted according to the method reported in reference [11]. Briefly, 9 g of wool (Corriedale) was immersed in 100 ml of water solution containing 8 m urea, 0.26 m SDS and 1.66 M 2-mercaptoethanol. The mixture was shaken at 50°C for 16 h and then filtered through stainless steel mesh. The filtrate was dialyzed against 3l of degassed distilled water using cellophane tubing (Union Carbide, Richmond, USA, No.30/32; molecular cut-off of about 10 kDa; diameter 2.5 cm) for 3 days changing outer water everyday. The protein concentration of dialyzate was 6.5% in average based on the Lowry method using protein assay kit (Sigma, St. Louis, USA). Thus, obtained keratin solution was stored at 4°C before use.

Preparation of Chitosan: Chitosan is a chitin derivate that is prepared by the N-deacetylation of chitin from the crustacean's shells. In general for preparation of chitosan first decalcificate shells with diluted HCl and deproteined with diluted NaOH and at the end deacetylated 40-50wt% of NaOH.

Preparation of Keratin-chitosan-gelatin Film: 250 mg of chitosan was stirred in 100 ml of 75% acetic acid at room temperature until homogenous solution was obtained. Three volumes of glacial acetic acid was added to the aqueous keratin-gelatin solution containing 100 mg of protein to give 75% acetic acid solution, which was then mixed with appropriate volume of chitosan solution (2.5 mg/ml).

To prepare composite of keratin-CS-gelatin we added a gelatin solution to keratin solution and then add various amount of chitosan to this composite it stirred in room temperature for about 2 hours over night and then remains it till it is become homogenous. Chitosan has varies contents in these experiments to evaluate the effects of chitosan on this composite Keratin-CS-gelatin. Composite was dissolved in a 1% (w/v) aqueous acetic acid solution with varies contents of chitosan at a concentration of 2% w/v. The solution was filtered through a glass filter to remove undissolved solids.

After drying, the transparent film was immersed in a coagulation bath containing 5% sodium hydroxide. The composite were washed with deionized water until the pH was 7.0 and then stored in de-ionized water. Then gelatin added to keratin-CS-gelatin composite. The gelatin content in the total solid was specifically 25%. The keratin-chitosan-gelatin solution was cast onto the polypropylene mold (bottom; 2×7 cm^2) and dried at 50°C overnight. The thickness of obtained film was 0.01-0.02mm on the average.

Mechanical Properties: Stress-strain curves were determined by uniaxial measurement using digital mechanical tester (Imada, Toyohashi, Japan). Films were cut into rectangle pieces (50×5mm^2) and measured at a crosshead speed of 20 mm/min at 25°C under 65% relative humidity (RH). The ultimate strength, the ultimate elongation and Young's modulus were calculated from five independent measurements.

We do our experimental works in Nov. 2007 to Feb. 2009 in laboratory of Islamic Azad University (Marvdasht Branch)

Scanning Electron Microscope of Chitosan Films: Surface morphology of the chitosan and keratin/ chitosan/ gelatin composite film was examined by scanning electron microscope.

Cell Culture: Human embryo lung diploid fibroblasts were cultured in MEM containing 11.5% bovine serum in an incubator with 5% CO2 at 37°C. When the cells completely covered the culture dish, they were detached using 0.25% pancreatin and collected by centrifugation. A cell suspension (20μL with 1000000 cells/mL) was added to each sample. After 2 h, 0.5mL of MEM medium was added and the culture trays maintained in an incubator with 5% CO2 at 37°C for 48 h. The adhesion and growth of the cells on material surface was observed with an inverted microscope. Then the cell was detached and the cell concentration was measured. The chitosan/keratin/gelatin composite film samples had 0, 10, 25, 33 and 50% gelatin content.

RESULTS

Preparation of Keratin-chitosan-gelatin Composite Film and its Mechanical Properties: To reinforce the film prepared by keratin, we tried to mix keratin with chitosan and gelatin that is soluble in water under acidic pH condition. Since wool keratin is an acidic protein whose isoelectric focusing point (pI) is between 4.9 and 6.1 [5], it becomes precipitated in a weakly acidic environment. Therefore, keratin-chitosan-gelatin were dissolved in 75% acetic acid and then mixed into a homogenous solution. The mixture was cast and dried to give a transparent film. The resultant film was stained with Coomassie Brilliant Blue (CBB) which is generally used for protein staining (data not shown). The staining occurred homogenously on the film, suggesting that keratin-chitosan-gelatin were perfectly mixed. The staining became weaker according to the increase of chitosan content. The mechanical properties of keratin-chitosan films are summarized in Table 1 and sketched in Fig1. When chitosan content was less than 5wt% of keratin and gelatin, the composite film was too fragile to measure its mechanical properties similarly to keratin film.

Comparison of Three Kinds of Composites

Scanning Electron Microscope Results: Aqueous chitosan/keratin solutions were transparent, hydrophilic and elastic. Gelatin is a water soluble polymer readily miscible with chitosan. Scanning electron microscope shows that the microstructure of pure chitosan film appears fabric-like while the CS/keratin/gelatin system was homogeneous and there was no obvious phase disengagement or macroscopic imperfection on the film.

Table 1: Effect of chitosan addition on mechanical properties of keratin-CS and glycerol-containing keratin

Chitosan (mg)	Ultimate Tensile Strength of keratin-CS(MPa)	Ultimate Tensile Strength of glycerol-containing keratin (Mpa)	Ultimate Elongation of keratin-CS (%)	Ultimate Elongation of glycerol-containing keratin (%)	Young's modulus of keratin-CS (Mpa)	Young's modulus of glycerol-containing keratin (MPa)
5	ND[a]	3±1	ND[a]	24±4	ND[a]	14±7
10	27±8	9±3	4±2	31±3	152±76	80±5
15	31±7	12±2	9±3	27±3	160±76	86±41
20	34±1	13±5	7±4	24±2	176±82	92±28
30	27±4	14±3	5±2	18±4	310±86	111±25

a. Not determined because of the fragility

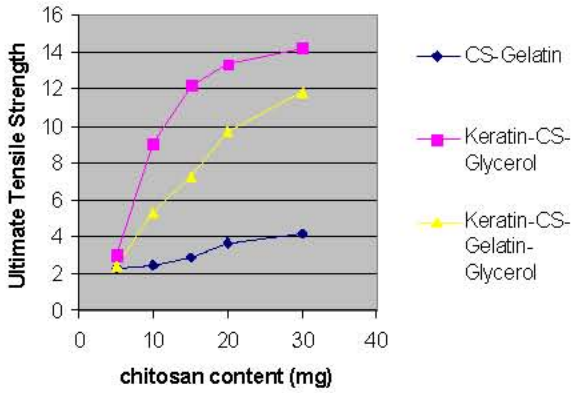

Fig. 1: Comparison of three kinds of composites

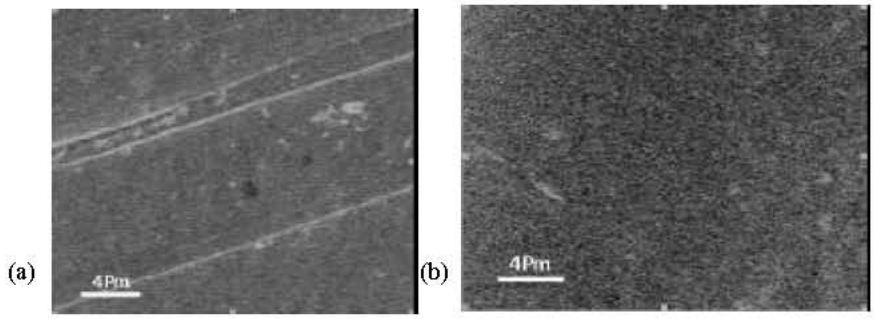

Fig. 2: SEM photos of (a) CS film and (b) Keratin/CS/gelatin composite film

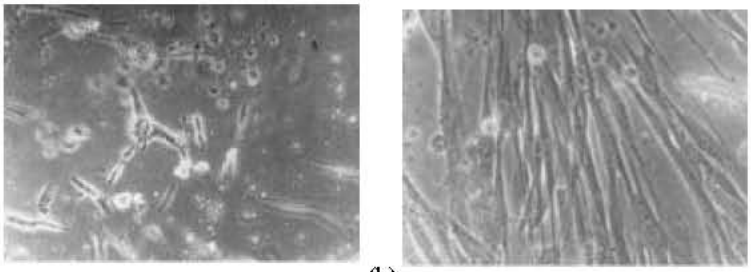

Fig. 3: Cell growth adhesion to films: (a) cell culture at 37°C for 48 h on chitosan film (b) cell culture incubated at 37°C for 24 h on the composite film

The CS/keratin/gelatin composite films were more flexible than the pure CS/keratin film. The SEM photos of chitosan film and keratin/chitosan/gelatin composite film are shown in Fig. 2.

Cell Cultured Results: Cell cultures are usually used in biomaterials research and development in order to detect toxicity of materials. It is an accurate and reliable method to test the biological compatibility of materials to be implanted *in vivo*. In this study, we investigated the cellular adhesion and growth on the surface of the chitosan films. Adhesion in the cell morphology after 2 h, the cells on the film appear spherical. After cell culture was incubated at 37°C for 24 h, it can be seen that cells have grown and spread on the film. The cells are arranged directionality and appear fibrous and during cell growth, they adhered to the films. The Cell growth and adhesion to chitosan and composite film are shown in Fig. 3.

The differences in cell adhesion to different gelatin content in the film may be related to the material surface characteristics such as surface charge. Inducing gelatin into chitosan film made the surface charge of the film become more negative than the CS film. Generally, the cell surface is negatively charged; therefore, the electrostatic attraction between cells and the CS/keratin/gelatin composite film is weaker than that between cells and CS film.

Table 2: Ultimate tensile strength of chitosan-gelatin and keratin-CS-gelatin containing glycerol with various chitosan contents

Chitosan (mg)	Ultimate Tensile Strength of chitosan-gelatin (MPa)	Ultimate Tensile Strength of keratin-CS-gelatin containing glycerol (MPa)
5	2.3	2.4
10	2.5	5.3
15	2.9	7.2
20	2.7	9.7
30	4.18	11.8

DISCUSSION

However, the film prepared from keratin-gelatin mixed with 10 wt% chitosan was fairly flexible and strong judged from ultimate strength (27±8 MPa), ultimate elongation (4±2%) and Young's modulus (152±76MPa). The further increase of chitosan content gave little additional change on ultimate strength and elongation, however, a little increased Young's modulus, suggesting that chitosan addition made a film stiffer. Gelatin and glycerol also gives high flexibility and low strength to the keratin film. As shown in Table 1 and 2, keratin film supplemented with 20 wt% of glycerol showed 3±1MPa of ultimate strength, 24±4% of ultimate elongation and 14±7MPa of Young's modulus. Addition of more than 10wt% chitosan also enhanced the ultimate strength (9-14MPa) and Young's modulus (80-111 MPa) of glycerol-containing keratin film, while ultimate elongation was not affected. As shown in Table 2 ultimate tensile strength of chitosan-gelatin is 2.3 for 5wt% of chitosan and various from 2.5 to 4.18 with different contents of chitosan. Chitosan-gelatin composite has low mechanical properties. With composite keratin-chitosan containing glycerol with gelatin we can improve mechanical properties of keratin chitosan composite to have more flexibility. As shown in Table 2 the ultimate tensile strength after adding gelatin to keratin-chitosan composite changes from 2.4 to 11.8. Fig. 1 is a comparison between ultimate strength of CS-gelatin, keratin-CS-glycerol and keratin-CS-gelatin. From these results, chitosan was found to increase mainly the strength and the stiffness of keratin film, while gelatin and glycerol is rather effective on flexibility. Therefore, appropriate addition of chitosan; gelatin and glycerol could control the mechanical properties of keratin film.

A composite film prepared from the mixture of keratin, chitosan and gelatin showed superior characteristics comparing with keratin and chitosan films, respectively. A film prepared from keratin was too fragile to handle without any additive. Although glycerol addition was reported to give a transparent flexible film [12], glycerol dissolved out in an aqueous environment. In the present work, we used chitosan as the additive to keratin film to reinforce the mechanical properties. Chitosan gave strength and flexibility to the keratin film. Comparing with chitosan film, keratin-chitosan-gelatin composite film had softness judging from Young's modulus. Keratin-chitosan-gelatin composite film showed remarkably improved waterproof characteristics. Keratin-chitosan-gelatin composite film was also shown to possess antibacterial activity in a similar fashion to chitosan film and to be a good substrate for mammalian cells. Keratin-CS-gelatin film is more effective in lens fabrications and implants because of having both flexibility and good tensile strength.

The CS/keratin/gelatin is more flexible than pure chitosan and chitosan/ keratin composite films and CS/keratin/gelatin films are homogeneous and no macroscopic imperfection seen on these films.

The cell cultured result show that after 48h, cell adhesion was a little greater on the CS/keratin/gelatin composite films than on the chitosan. The number of anchored cells on CS/keratin/gelatin films was also greater. The CS film appears to be the films were inferior to the CS/keratin/gelatin film in cell adhesion and growth.

CONCLUSION

Chitosan forms elastic and transparent films that have excellent hydrophilic properties. The keratin-CS-gelatin composite system has good biocompatibility and antibacterial properties. By increasing the gelatin content, the hydrophilicity, solute and oxygen permeability of the CS films were improved [17]. In keratin-CS-gelatin composite films, chitosan plays an important role for mechanical strength while increasing the CS content enhanced the mechanical strength of the composite film. The biocompatibility of chitosan film; the diploid fibroblast cell growth and their adhesion to films were better on the CS content films than on the contact lens, implants and other applications. In previous research use gelatin to prepare lens or use only chitosan for implants but in this research we use a composite that covers the defects of using chitosan, gelatin or keratin alone, as mentioned above.

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