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# Effect of glutaraldehyde on bioadhesive strength between rat skin and biofilm prepared from yellowfin tuna (*Thunnus albacares*) gelatin

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**Abstract**−Bioadhesive strength between biofilm from yellowfin tuna (*Thunnus albacares*) gelatin and rat skin treated with glutaraldehyde was compared with bovine and porcine gelatin. Tuna biofilms treated with 0.5 M glutaraldehyde at 60 °C and neutral pH for 2 h had an increase in bioadhesive strength of approximately five times when compared with intact tuna biofilms. When glutaraldehyde-treated tuna biofilms were applied with sodium borohydride, the bioadhesive strength was reduced to the level of intact tuna biofilms. The bioadhesive strength of tuna biofilms was superior to those of bovine and porcine gelatin biofilms.

Key words: Biofilm, Bioadhesion, Yellowfin Tuna, Gelatin Film, Glutaraldehyde

## INTRODUCTION

Bioadhesion is the process of linking natural and synthetic polymers with a target bio-component, which is manifested by factors such as mucus adhesion, hydrogen bonding, salt bridging, ionic bonding and various others [1]. Bioadhesive characteristics in the target tissue of derma are formed by interrelated forces of atoms and molecules on the surfaces between the polymer and target [2]. Important factors that affect bioadhesive capacity are surface energy and contact angle [3,4], mutual infiltration between polymer chains, the rate of expansion by interaction between polymer and stringy proteins, and the epidermis [5]. Bioadhesive material can be divided into natural and synthetic polymers [6]. Synthetic polymers are carbomers of poly(acrylates), poly(acrylic acid), polycarbophil and cyanoacrylate derivatives [7,8]. Natural polymer bioadhesives are typically polysaccharides, cellulose derivatives [9], hyaluronic acid [10], chitosan [11] and gelatin [12]. Important factors required of bioadhesives are low stimulus, low toxicity, adequate fluidity and biodegradability of the target tissue.

Collagen-derived gelatin has biodegradability, biocompatibility, heat-reversible sol-gel formation, water holding capacity, binding property, emulsifying capacity, cohesion, and viscosity, and thus can be used in several areas including the food, medicinal, and film industries [13]. Gelatin has superior adhesive strength compared with fibrin glue, which is commonly used in the process of cross-linking in medical adhesives [14]. Generally, the physicochemical properties of fish gelatins are known to be inferior to those of mammalian gelatins, and have little commercial value [15]. Bovine and porcine gelatins, which are used mainly in commercial products, reveal the limitations for the application of nutraceuticals, cosmetics, and pharmaceuticals because of possible contamination by bovine spongiform encephalopathy (BSE) or foot-and-mouth diseases (FMD) [16]. Fish gelatins obtained as byproducts during fish processing may be useful as replacements for the mammalian materials.

Approximately 3.4 million tons of yellowfin tuna are harvested annually, and large amounts of gelatin can be obtained from tuna skin (containing 12% gelatin). Tuna gelatin is also safer than mammalian gelatin with regard to BSE and FMD. Some of the physicochemical properties of yellowfin tuna dorsal skin gelatin are even superior to bovine and porcine gelatins [16]. Also, the adhesive strength of tuna gelatin to plywood has been shown to be superior to that of bovine and porcine gelatins [17].

The objectives of this study were to investigate the adhesion properties of the glutaraldehyde modified tuna gelatin film for use as a bioadhesion material. Glutaraldehyde has two aldehyde groups per molecule, and each can form the strong Schiff base through an amino-carbonyl reaction with the free amino groups of proteins, such as gelatin. Either of the two aldehyde groups can cross-link with amino groups of proteins over variable distances, and therefore the potential for cross-linking of glutaraldehyde is greater than with monocarbonyls, such as formaldehyde. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size [18]. There is a free aldehyde group protruding from the side of each unit of the polymer molecule, as well as one at each end. These aldehyde groups can combine with free amino groups of proteins with which they come into contact, so there is enormous potential for cross-linking [19].

In this study, we investigated the bioadhesive properties of the yellowfin tuna gelatin biofilm with rat skin for use as a bioadhesive material. The bioadhesive properties are also compared with bovine and porcine skin gelatins.

## EXPERIMENTAL SECTION

### 1. Materials

Gelatin was extracted from the dorsal skin of yellowfin tuna (*Thunnus albacares*) and purified according to the method of Cho et al. [16]. Rat skin was obtained from 4-week old Sprague-Dawley male white rats. Chitosan from crab shells was purchased from Sigma

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Chemical Co. (EC No 222-311-2, Sigma, USA). Bovine gelatin (EC No 232-554-6, Type B, Sigma), and porcine gelatin (EC No 232-554-6, Type A, Sigma) were purchased from Sigma Chemical Co. (USA). Additional chemicals used in this study were 25% glutaraldehyde solution, 2-mercaptethylamine hydrochloride, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), sodium borohydride, sodium bicarbonate which were purchased from Sigma Chemical Co. (USA). All other chemicals for this study were analytical grade.

## METHODS

### 1. Processing of Gelatin Biofilm

Gelatin solution (6.67%, 225 mL) was put in the 20×30 cm plastic mold and dried at 25±1 °C and 50±1% relative humidity (RH) for 24 h to produce an experimental biofilm of 0.001 mm film thickness.

### 2. Treatment of Tuna Gelatin with Glutaraldehyde

The processed biofilm was cut to 1×1×0.01 cm. For treatment with glutaraldehyde, cut films were placed into 50 mL of phosphate-citrate buffer solution (pH 7) containing 0.5 M glutaraldehyde. The treatment was performed at 60 °C for 2 h. Next, the biofilm was washed twice with distilled water and dried at ambient temperature for 24 h.

### 3. Measurement of Bioadhesive Strength

Bioadhesive strength between gelatin biofilm and rat skin was measured as described previously (initially used to measure fibrin glue-bioadhesive strength) with slight modifications [18]. Rat skin was stored under vacuum at −18 °C for a maximum of 2 weeks. For experimental usage, the frozen rat skin was thawed and cut to the size of 1×3 cm and 1 mm thickness using a scalpel. Adhesive strength was measured at 25±2 °C and 50±2% RH. The 1×1 cm dried gelatin biofilm was put on a 1×3 cm derma edge of a piece of rat skin. Another piece of rat skin was placed on the opposite side of the biofilm, making the adhesion areas 1×1 cm on both sides. A force of 1 kg was applied to the adhesion area for 10 min and the bioadhesive strength was measured with a Universal testing machine (Instron 1011, USA). The bioadhesive strength was defined as the maximum load value at the complete separation of the adhesion area. Measurements were replicated five times.

### 4. Measurement of Free Aldehyde Groups

The 5×5 mm gelatin biofilm was put into 1 mL aqueous solution of 0.1 M 2-mercaptoethylamine hydrochloride at 37 °C for 8 h to convert aldehyde groups to thiol groups. The biofilm was then placed into 2 mL carbonate-buffered solution (pH 10) of 2.5% sodium borohydride at 37 °C for 1 h to reduce Schiff bases. It was then washed twice with phosphate-citrate-buffered solution (pH 7). The biofilm was put into 5 mL phosphate-citrate buffer solution (pH 7) of 1 mM (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) at 37 °C for 15 h under nitrogen gas to facilitate the exchange reaction of thiol-disulfides. The absorbance of the solution of free thionitrobenzoate anion (**II**) was measured at 412 nm using a UV spectrophotometer. The concentration of aldehyde groups was calculated by multiplying the absorbance by 13,600 (absorption coefficient of thionitrobenzoate anion) (**II**) [20].

### 5. Treatment of the Biofilm with a Reducing Agent

The biofilm (1×1×0.001 cm) was reacted in each solution of 0.1-1.0 M glutaraldehyde at 60 °C for 2 h, and then was treated with 50 mL solution (pH 10) of 2.5% $NaBH_4$ at 60 °C for 15 min.

### 6. Statistical Analysis

The experimental data are presented as means±one standard deviation. Significant difference tests were performed by analysis of variance (ANOVA) using the SAS program at the probability level of $\alpha$=0.05. The multiple comparisons were performed using Duncan's multiple test at the probability level of $\alpha$=0.05.

## RESULTS AND DISCUSSION

### 1. Effect of the Glutaraldehyde Concentration

There are two aldehyde groups per glutaraldehyde molecule, and each can form the strong Schiff base through an amino-carbonyl reaction with the free amino groups of proteins, such as gelatin. Either of the two aldehyde groups can cross-link with amino groups of proteins over variable distances, and therefore the potential for cross-linking of glutaraldehyde is greater than with monocarbonyls, such as formaldehyde. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size [18]. There is a free aldehyde group protruding from the side of each unit of the polymer molecule, as well as one at each end. These aldehyde groups can combine with free amino groups of proteins with which they come into contact, so there is enormous potential for cross-linking [19].

Cross-linking of proteins by glutaraldehyde can result in improved physicochemical properties, such as gel strength and bioadhesive strength. Fig. 1 shows bioadhesive strength to rat skin, and aldehyde content of the tuna gelatin biofilms treated with different concentrations of glutaraldehyde. The bioadhesive strength of tuna gelatin biofilms was strongly increased by addition of glutaraldehyde. As glutaraldehyde concentration was increased from 0.1 M to 1.0 M, the bioadhesive strength was increased from 16.33 kPa to 32.86 kPa. There was no significant difference at concentrations higher than 0.5 M glutaraldehyde. As glutaraldehyde concentration increased from 0.1 M to 1.0 M, the concentration of aldehyde groups in the biofilm was increased from 4.62 mM/g to 6.01 mM/g. Thus, the

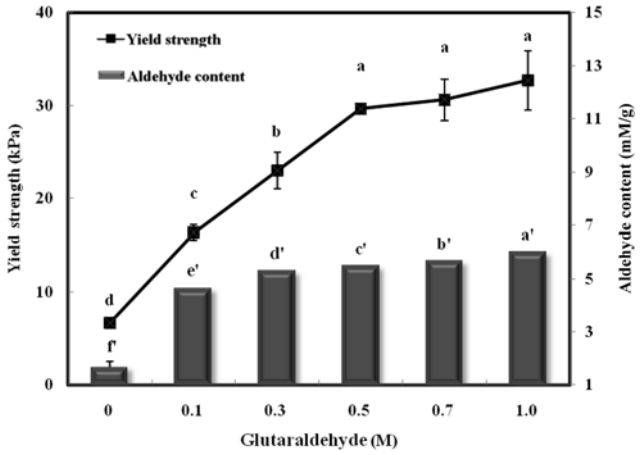


**Fig. 1. Analysis of the effects of increased aldehyde concentrations on the bioadhesive strength between rat skin and yellowfin tuna gelatin biofilms through addition of varying glutaraldehyde concentrations for 2 h at 60 °C. Different letters (a, b, c, a′, b′, c′, etc.) indicate significant differences at the $\alpha$ level of 0.05.**

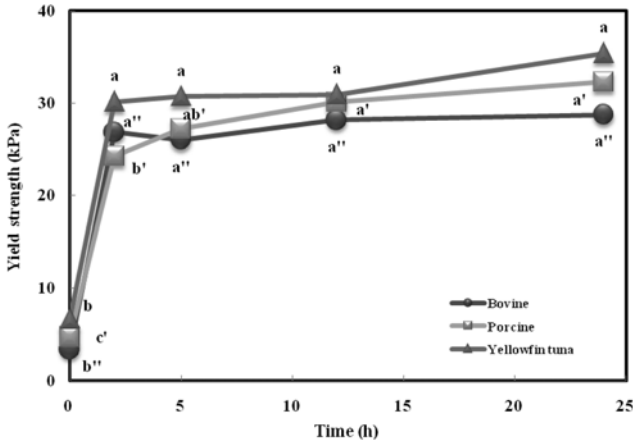


**Fig. 2. Bioadhesive strength of yellowfin tuna, bovine and porcine gelatin biofilms treated with 0.5 M glutaraldehyde for different times at 60 °C. Different letters (a, a′, a″, etc.) indicate significant differences at the $\alpha$ level of 0.05.**

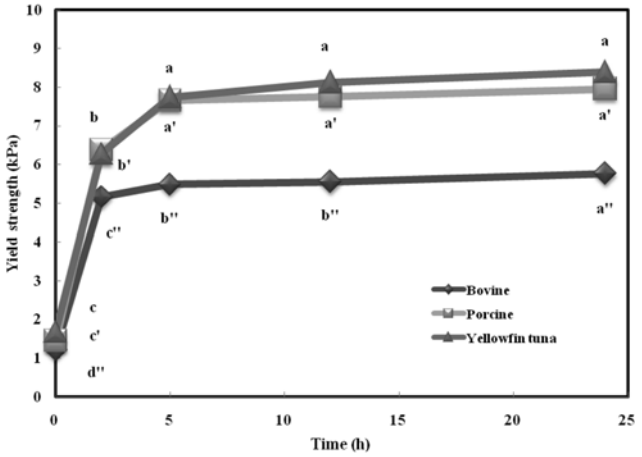


**Fig. 3. Aldehyde content of the yellowfin tuna, porcine, and bovine gelatin biofilms treated with 0.5 M glutaraldehyde for a varying times at 60 °C. Different letters (a, a′, a″, etc.) indicate significant differences at an $\alpha$ level of 0.05.**

cross-linking between the aldehyde and the amino groups in the glutaraldehyde-treated biofilm of rat skin increased bioadhesive strength.

Gelatin for bioadhesive has carboxyl group in molecules. Carboxyl group of bioadhesive materials can be ionized between the molecules of the carboxyl group and the one of amino or hydroxyl group to form multiple bonds. Multiple combinations of carboxyl group among the adhesive and amino acid between molecules makes to bond easily [12]. Accordingly, it is considered that adhesive strength of gelatin film is due to cross linking with aimed-group and multiple bonds among the carboxyl group and the one of amino or hydroxyl group.

### 2. Effect of Altering Exposure Time to Glutaraldehyde

Changes in the bioadhesive strength of the tuna gelatin biofilm under different exposure times to glutaraldehyde are shown in Fig. 2. Changes in the bioadhesive strength of tuna gelatin biofilm during the treatment time were compared with those of biofilms from the bovine and porcine gelatin, which are used most commonly for industrial applications in foods and medicines. The bioadhesive strengths of tuna, bovine, and porcine gelatin biofilms were significantly increased by addition of glutaraldehyde. The tuna gelatin biofilm showed higher bioadhesive strength shortly after exposure, while bovine and porcine gelatin biofilms reacted more slowly. After 2 h of treatment time, the increased strength of tuna gelatin biofilm was such that it was not significantly different from bovine or porcine gelatin. The altered pattern of aldehyde groups in the tuna gelatin biofilm with treatment time (Fig. 3), was similar to the changes in the bioadhesive strength.

### 3. Effect of pH and Temperature During Glutaraldehyde Treatment

Bioadhesive strength using rat skin and tuna gelatin biofilm treated at several pHs in 0.5 M glutaraldehyde solution are shown in Fig. 4. At pH 3, the bioadhesive strength was lowest (15.60 kPa), while it was highest at pH 7 (31.09 kPa). As the pH shifted towards alkaline, the bioadhesive strength decreased to 22.95 kPa. While studying gelatin extraction at a pH range of 3-10 at 50 °C for 2 h from conger skin, physical properties such as gel strength, melting point, gelling point, and color were improved as the extraction pH was

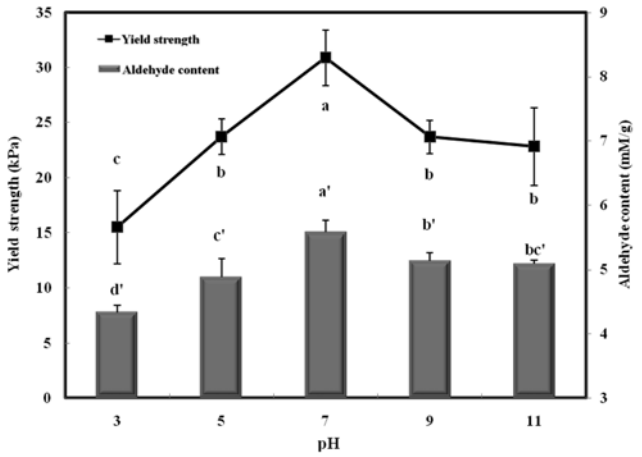


**Fig. 4. Bioadhesive strength between rat skin and the aldehyde content of the yellowfin tuna gelatin biofilms treated with 0.5 M glutaraldehyde of different pHs for 2 h at 60 °C. Different letters (a, b, c, a′, b′, c′, etc.) indicate significant differences at an $\alpha$ level of 0.05.**

increased up to pH 6 and 7 [21]. In extractions at pHs higher than 7, the qualities have been shown to decrease. During gelatin extraction, the gelatin was quickly changed to the reduced form by heating. The extraction rate was lowest at neutral pH, followed by alkaline pH and acidic pH. The physical properties of gelatin were reduced by the significantly decreased molecular weight, when gelatin was heated at acidic or alkaline pHs. Treating the gelatin biofilm at several pHs in a glutaraldehyde solution of 0.5 M at 60 °C for 2 h resulted in reduced molecular weight by heating in acidic or alkaline conditions. The original properties of the gelatin biofilm were lost, so the bioadhesion strength was thought to decrease. Thus, the bioadhesive strength of gelatin biofilm treated in several different pH solutions of 0.5 M glutaraldehyde at 60 °C for 2 h were reduced. That the gelatin molecules were quickly degraded to lower molecular weight states in acidic conditions versus alkaline conditions is consistent with the bioadhesive strength being reduced in acidic conditions. The con-

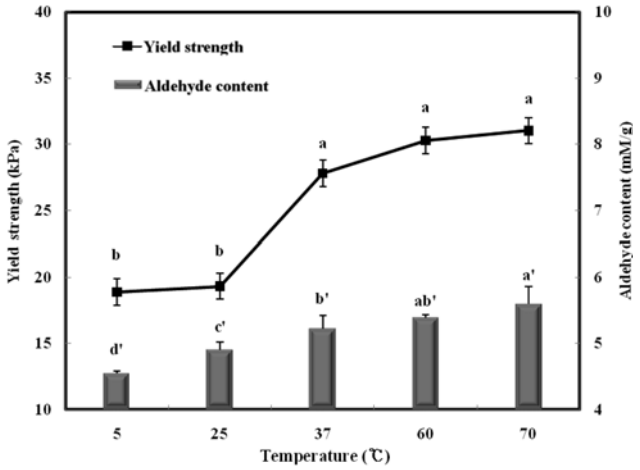


**Fig. 5. Bioadhesive strength between rat skin and aldehyde content of the yellowfin tuna gelatin biofilms treated with 0.5 M glutaraldehyde for 2 h at different temperatures. Different letters (a, b, c, a′, b′, c′, ...) indicate significant differences at an $\alpha$ level of 0.05.**

centration of aldehyde groups in glutaraldehyde-treated gelatin biofilms showed higher values (5.58 mM/g) at neutral pH and lower values at acidic and alkaline pHs (4.33 and 5.09 mM/g, respectively).

The bioadhesive strength of rat skin with the gelatin biofilm treated at several temperatures with 0.5 M glutaraldehyde solution of neutral pH is shown in Fig. 5. Bioadhesive strength increased with temperature. The strength increased gradually until 25 °C, and sharply increased from 37-60 °C. Temperatures above 60 °C did not result in increased bioadhesive strength. Changes in the concentration of aldehyde groups followed the same pattern as the increase in bioadhesive strength. Based on these results, the increased bioadhesive strength of the glutaraldehyde-treated tuna biofilm is likely due to the increased number of aldehyde groups.

**4. Effect of a Reducing Agent on Bioadhesive Strength**

Sodium borohydride was used as a reducing agent for the aldehyde groups of glutaraldehyde, which likely affect the bioadhesive properties of the films. A comparison of the bioadhesive strengths of the films before and after treatment with sodium borohydride is shown in Fig. 6. As glutaraldehyde concentration increased, the bioadhesive strength of the biofilm increased. This increase was inhibited through treatment with the reducing agent sodium borohydride. The decrease in bioadhesive strength by treatment with a reducing agent resulted in a decrease in free aldehyde groups that can react with the amino groups within rat skin.

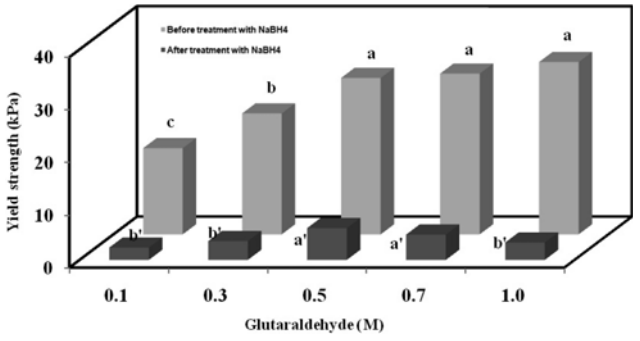


**Fig. 6. Effect of the reduction of aldehyde groups in the glutaraldehyde-treated yellowfin tuna gelatin biofilms on their bioadhesive strength with rat skin. The biofilms were subjected to glutaraldehyde treatment at 60 °C for 2 h in glutaraldehyde solutions of different concentrations. Next, the biofilms were treated with 2.5% $NaBH_4$ at pH 10 for 15 min. Different letters (a, b, c, a′, b′, c′, etc.) indicate significant differences at the $\alpha$ level of 0.05.**

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