Low cytotoxic tissue adhesive based on oxidized dextran and epsilon-poly-L-lysine

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Abstract

A novel adhesive hydrogel consisting of dextran and epsilon-poly(L-lysine) (dextran-PL) with multiple biomedical applications was developed. Periodate oxidation in aqueous media almost stoichiometrically introduces aldehyde groups in dextran molecules, and aldehyde dextran can react with the primary amino groups in epsilon-PL (ε-PL) at neutral pH to form a hydrogel. The gelation time of the hydrogel can be easily controlled by the extent of oxidation in dextran and of the acylation in ε-PL by anhydrides. The shear adhesion strength of dextran-PL was ten times higher than that of fibrin glue, when wet collagen sheets were selected as test specimens. The cytotoxicity of aldehyde dextran and ε-PL were 1,000 times lower than that of glutaraldehyde and poly(allylamine). The considerably low cytotoxicity of aldehyde dextran could be ascribed to its low reactivity with amine species when compared with glutaraldehyde. In contrast, a high reactivity of amino groups in ε-PL was observed when compared with glycine, L-lysine, and gelatin, which could be explained by their poor dissociation at neutral pH, thus leading to low cytotoxicity.

1. Introduction

Various types of surgical tissue adhesives have been developed to fix the tissue together (glues), to control bleeding (hemostats), and to close off air or liquid leaks (sealants), which are composed of synthetic or biological compounds, or their combinations. Cyanoacrylates are very familiar synthetic glues, which rapidly polymerize on contact with water or blood. *N*-butyl-2-cyanoacrylate (Histacrylblue™, B. Braun, Melsungen, Germany) and 2-Octyl-cyanoacrylate (Dermabond™, Ethicon Inc., Somervillen, NJ, USA) are commercially available and used in endoscopic therapy and plastic surgery.[1,2] Cyanoacrylates have a strong adhesive strength, however, they are synthetic and absorbable, and make an impenetrable and rigid barrier without elasticity. In clinical use, systematic inflammatory responses[3] and poor handling[4] were reported, and high cytotoxicity[5] has also been observed. Photochemically crosslinking poly(ethylene glycol) is another example of a synthetic adhesive (Focalseal™, Genyzme Biosurgery Inc., Cambridge, MA, USA, FDA approved) that is used in cardiac surgery, which requires a photosource and long crosslinking time.[6]

Fibrin sealant or fibrin glue, one of the biological glues, is the most widely used in clinical applications, and consists of two components, a highly purified human fibrinogen with factor XIII and a human thrombin solution. Various brands, such as Tisseal™ (Baxter, Westlake Village, CA, USA) and Bolheal™ (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) are commercially available. Fibrin sealants have the advantage of biocompatibility and biodegradability compared with synthetic sealants. As the degradation of the sealant is a part of the normal wound healing process, no inflammation, foreign body reactions, tissue necrosis, or extensive fibrosis have

been reported.[7] Fibrin glues have been used mainly for hemostasis in vascular surgery[8] gastrointestinal endoscopic therapy[9] and other applications, such as for enterocutaneous fistulas[10] and esophageal perforations.[11] Some complications associated with fibrin glue have been reported, such as serious bleeding diatheses[12] and weak adhesion.[13-15] In addition, a risk of infectious transmission due to the biological origin was widely reported.[12,16-18] Another type of biological glue is a combination of bovine thrombin and bovine collagen (Floseal™, Sulzer Spine-tech, Anaheim, CA, USA), and is used for hemostasis in vascular surgery.[15,19] Similar to fibrin glue, this bovine-derived sealant is also not free from the risk of viral infection.

A combination of synthetic and biological components is represented by gelatin-resorcinol-formaldehyde-glutaraldehyde (GRF) glue (Medico Corp., Bucuresti, Romania). Currently, Bioglue™ (Cryolife, Inc., Kennesaw, GA, USA) is approved for use in the United States, which is a combination of bovine albumin with glutaraldehyde, and is used in otolaryngology,[20] urology,[21] and aneurological surgery.[22] However, the strong inflammatory response[23,24] and high toxicity[25] caused by the release of formaldehyde and glutaraldehyde have made their clinical applications limited to the repair of aortic dissections.[26] So far, none of these adhesives fulfills all a clinician's requirements, and therefore, a great deal of effort has been made to prepare new synthetic or semi-synthetic ideal adhesives. Generally, biological molecules are bioactive and play important roles in cell proliferation, migration, and differentiation, which is an advantage with regard to the molecular design of the adhesive, compared with synthetic molecules. Therefore, developing adhesives consisting of both biological and synthetic components has been of great interest.

In our preliminary study, epsilon-poly(L-Lysine) (ε-PL) was used in combination with aldehyde polysaccharides.[27] Epsilon-PL, an L-lysine homopolymer biosynthesized by *Streptomyces* species, in which epsilon-amino groups and alpha-carboxyl groups form repeating amide bonds (as is shown in Figure 1), is currently used as a food additive due to its antimicrobial activities.[28,29] The preparation of a ε-PL hydrogel was recently reported.[30] Dextrans, biosynthesized by *Leuconostoc mesenteroides* species, are glucose polymers which have been used clinically for more than 5 decades for plasma volume expansion, peripheral flow promotion, and as antithrombolytic agents.[31] Dextran is biocompatible[32] and can be degraded through the action of dextranases in various organs in the human body, including the liver, spleen, kidneys, and colon.[31] In this publication, we report the synthesis of hydrogels consisting of ε-PL with dextran and characterize their adhesive properties. In particular, we focused on the cytotoxicity of the hydrogels forming materials, aldehyde dextran and ε-PL, to determine their potential for future clinical applications.

2. Materials and methods

Dextran with a molecular weight of 70K Da was obtained from Meito Sangyo Co. Ltd., (Nagoya, Japan). Epsilon-PL (4K Da, aqueous 25 w/w% soltion, free base) was from JNC Corp. (Tokyo, Japan). Fibrin glue (Bolheal™) was from Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. Sodium periodate, acetic anhydride, succinic anhydride, dextrin, and other chemicals were from Nacalai Tesque, Inc., (Kyoto, Japan) and used without further purification, unless otherwise mentioned.

2.1 Oxidation of dextran with periodate

Aldehyde dextran was prepared by the oxidation of dextran with sodium periodate according to the method reported by Mo *et al* .[32] Briefly, 20 g of dextran was dissolved in 80 mL of distilled water, and different amounts of sodium periodate (0-10 g) were dissolved in 40 mL of water. Then, both of the solutions were mixed together and the reaction proceeded at 50ºC for 1 h under gentle stirring. The reaction mixture was dialyzed against running water (1 L/min) for 16 h, and distilled water (3 L, 1.5 h, 2 times) using a cellulose dialysis membrane (cut off molecular weight of 14,000, Viskase Companies, Inc., Darien, IL, USA). Aldehyde dextran was recovered by air-drying for 48 h at 40ºC and vacuum drying for 24 h at 25ºC. The obtained products were mechanically crushed into a fine powder and dried thoroughly for 24 h at 50ºC under a vacuum, and were stored at –20ºC until use.

The aldehydes introduced in dextran were evaluated by simple iodometry. Briefly, 10 mL of approximately.1 w/v % aqueous aldehyde dextran solution (dissolved in distilled water at 60ºC, cooled down to 25ºC, and diluted to an appropriate volume with water) was added to 20 mL of I_2 solution (0.05 mol/L), followed by addition of 20 mL of NaOH (1 mol/L). The oxidation reaction proceeded for 15 min at 25ºC. After the addition of 15 mL of H_2SO_4 (6.25 v/v%), the I_2 consumption by the reaction with aldehyde was titrated with 0.1 mol/L of $Na_2S_2O_3$ using a drop of aqueous 20 w/w% of dextrin solution as an indicator, where one mole of aldehyde groups reacts with one mole of I_2 under alkaline conditions, thus leading to the formation of carboxyl acid, and one mole of I_2 reacts with 2 moles of $S_2O_3^{2-}$ ion. Triplicate readings were taken for each titration (n = 3).

2.2 Acylation of ε-PL by anhydrides

Epsilon-PL, an oligomer of L-lysine, has about 30 primary amino groups per molecule. To change the gelation, some of the amino groups were acylated by the addition of carboxylic anhydrides. Four g of 25 w/w% aqueous ε-PL solution, different amounts of acetic or succinic anhydride, and distilled water were mixed, and the reaction was allowed to proceed for 1 h at 50°C under gentle stirring, where the ε-PL concentration in the reaction was 10 w/w% in each reaction. The acylated ε-PL were purified by dialysis (cutoff molecular weight: 10 kDa, Spectra/Por, Spectrum Laboratories, Inc., CA, USA) against water for 24 h and then freeze-dried for 24 h. The chemical structures of the compounds were confirmed by ^{1}H NMR (400 MHz, Bruker) using D_2O as solvent. The amino groups remaining in ε-PL after acylation were evaluated by the ninhydrin method.[34] The acylated ε-PL solution (10 w/w%) was diluted by 400 times with distilled water, and then 0.1 mL of the dilution, 1 mL of ninhydrin solution (0.8 g of ninhydrin and 0.12 g of hydrinedantin anhydrous in 30 mL of 2-methoxyethanol), and 2 mL of acetate buffer solution (0.1M acetic acid and 0.2M sodium acetate, pH 5.5) were added to a glass tube, followed by heat sealing. After colorization for 3 min at 100°C, then the absorbance at 570 nm was recorded at 25°C (spectrophotometer U-2800A, Hitachi Co. Ltd., Tokyo, Japan). Triplicate readings were taken for each sample (n = 3).

2.3 Gelation time measurement

The mixture of aqueous aldehyde dextran and ε-PL easily formed a hydrogel, and the gelation time

was evaluated by a simple stirring method.[35] A 0.5 mL aliquot of 20 w/w% of the aqueous aldehyde dextran was added to a glass tube (16 mm in diameter) and incubated for 10 min at 37ºC, and then 0.5 mL of 10 w/w% ε-PL solution at 37ºC was added to the tube. In this mixing ratio, the pH of the mixture was around 7 in all cases. The period of time until a small magnetic stirring bar (4 x 10 mm) was stopped by gel formation was recorded (the speed of stirring was 280 r.p.m. using a Mighty Magnetic Stirrer M-12G6, Koike Precision Instruments Co. Ltd., Hyogo, Japan). Triplicate readings were taken for each sample (n = 3).

2.4 Bonding strength evaluation for wet test specimens

The bonding strength of the dextran-PL tissue adhesive was measured according to the method reported by Kakinoki *et al*.[36] A collagen casing (#320, a tube that was 32 mm in diameter, Nippi Inc., Tokyo, Japan.) was cut into 80 x 15 mm pieces and stored in distilled water at 4ºC until the measurement. After gently wiping out the surface water, approximately 0.1 mL of dextran-PL consisting of the same volume of 20 w/w% aldehyde dextran (22.6% oxidation) and 10 w/w% ε-PL containing 2 /w/w% of acetic anhydride was dropped onto one piece of the swollen test specimen, and then another specimen was put on it with a bonding area of 15 mm x 15 mm. After 2 min with loading of 200 g, the shearing bonding strength was measured at a crosshead speed of 10 mm/min, using a tensile strength machine (RTC-1210A, A&D Co. Ltd., Tokyo, Japan). To mix dextran and ε-PL solution effectively, a dual syringe device was used for the test (Mini-Dual Syringe, 2 x 2 cc, Plas-Pak Industries, Inc., CT, USA, Figure 1). In this experiment, fibrin glue, Bolheal™, was also chosen

for a reference material and used according to the product insert and the manufacturer's instructions. For both adhesives, five specimens were tested (n = 5) at 25ºC.

2.5 Cytotoxicity testing

The cytotoxicity of aldehyde dextran and ε-PL was evaluated by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) method.[37] L929 (American Type Culture Collection, Manassas, VA, USA), an established mouse cell line, which has been often selected for cytotoxicity tests, was used and cultured in Eagles' MEM (Nissui Phermaceutical Co. Ltd., Tokyo, Japan) supplemented with 0.15 w/v% of hydrogen bicarbonate, 0.03 w/v% of L-glutamine, and 10 v/v% of fetal bovine serum. Cell culture was carried out at 37°C under 5% CO_2 in a humidified incubator. Cultured L929 cells in the logarithmic growth state were trypsinized and suspended in culture medium at a concentration of 1.0 x 10^4 cells/mL. Following the addition of 0.1 mL of the suspension to a 96-well tissue culture plate, the cells were incubated for 3 days at 37°C. Then, 0.1 mL of culture medium containing different concentrations of test substances was added to each well, followed by further incubation for 2 days. After discarding the medium and rinsing the cells with 0.2 mL of phosphate buffer saline (PBS) 3 times, 0.1mL of the MTT solution (90 mg of MTT dissolved in 100 mL of the culture medium) was added to the culture and incubated at 37ºC for 5 h. The formazan crystals in the culture plate were dissolved in 0.1 mL of dimethyl sulfoxide and the absorbance at 540 nm was recorded by a microplate reader (Versa Max, Molecular Device Japan K.K., Tokyo, Japan). The cytotoxicity of the test substances was expressed as the 50% inhibition concentration of growth

(IC50), which was defined as the concentration in the culture at which the cell activity was reduced to 50% of that of the untreated control cells, where 8 wells were used in both cases.

In this experiment, alpha-poly(L-lysine) (α-PL, hydrobromide, Mw 5,000-15,000, Wako Pure Chemical Industries Ltd., Osaka, Japan), poly(allylamine) (aqueous 20 w/w%, free base, Mw 5,000, Nitto Boseki Co. Ltd., Fukushima, Japan), glutaraldehyde (aqueous 25 w/w%), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, Dojin Chemical Laboratories Co. Ltd., Kumamoto, Japan), acetic acid, and succinic acid were also selected for comparison. All the test substances were dissolved in distilled water and filtration-sterilized with a membrane filter with a pore size of 0.22 μm, followed by dilution with the culture medium prior to addition to the cell culture.

2.6 Reactivity of aldehyde dextran and amino groups in ε-PL

The reactivity of the aldehyde in aldehyde dextran was compared with that of glutaraldehyde. Aqueous 25 w/v% of glutaraldehyde was diluted with 50 volumes of distilled water, so that the aldehyde concentration was 15.5 mM, which was estimated by the same method used in section 2.1. Aldehyde dextran with 22.6% oxidation was also dissolved in water at a concentration of 10 w/v%, where the aldehyde concentration was 29.8 mM. One mL of gelatin (MW 100K, isoelectric point 9, Nitta Gelatin Inc., Osaka, Japan) or bovine serum albumin solution (1 w/v% in PBS) and different amounts of aldehyde dextran or glutaraldehyde solution (0-1 mL) were put into glass tubes (16 mm in diameter). Then distilled water was added to the tube to give the total solution a volume of 2 mL, and the reaction was allowed to proceed for 3 h at 37ºC with gentle stirring. The amino groups

remaining in the gelatin or albumin were evaluated by 2,4,6-trinitrobenzenesulfonic acid (TNBS) method.[38] A 0.1 mL aliquot of the reaction mixture, 1 mL of aqueous TNBS solution (0.1 w/v%), and 2 mL of bicarbonate buffer (containing 4 w/v% of $NaHCO_3$ and 1 w/v% of sodium dodecyl sulfate, pH 9.0) were put into a glass tube (16 mm in diameter) and color development proceeded for 2 h at 37°C. The absorbance at 335 nm at 25°C was recorded, and the amino group consumption by the reaction was calculated. In this experiment, triplicate readings were taken for each sample (n = 3).

The reactivity of the amino groups in ε-PL was also compared with that of other simple amino species such as glycine, L-lysine, and gelatin (the same type used above). First, the amino group contents in gelatin originating from amino acids such as L-lysine was estimated by the TNBS method. When the same concentration of the solution (1 mg/mL) was prepared, the absorbance of glycine, lysine hydrochloride, and ε-PL were 22.64, 22.03, and 19.52 times higher than that of gelatin, respectively. Based on these values, test solutions having the same concentration of primary amine were prepared. Glycine or L-lysine hydrochloride were dissolved in phosphate buffer (0.075M of KH_2PO_4 and 0.225M of K_2HPO_4, pH 7.4) at concentrations of around 0.5 mg/mL. Gelatin was dissolved at 10 mg/mL. A 25 w/w% aqueous ε-PL solution was diluted 500 times with phosphate buffer to obtain a concentration of 0.5 mg/mL. Then, 2 mL aliquots of primary amine solutions of glycine, L-lysine, gelatin, and ε-PL were prepared according to the absorbability ratios to have 1.47 mM per glass tube (16 mm in diameter). To the tube, different volumes of aldehyde dextran solution (22.6% oxidation, 10 w/v%, 29.8 mM of aldehyde) and the phosphate buffer were added to reach a total volume of 4 ml. The reaction proceeded for 3 h at 37°C with gentle stirring. After the reaction,

the amino groups remaining were estimated in the same manner described above. Here, the ratio of the molar concentration of aldehyde to amino groups in the reaction varied from 1/1 to 50/1. Triplicate readings were taken for each sample (n = 3).

2.7 Statistical analysis

All data are expressed as the mean ± standard deviation (SD). The Student's t-test was used to make comparisons between 2 groups. Data among more than 3 groups were compared using a one-way analysis of variance (ANOVA) with a post-hoc Tukey–Kramer test.

3. Results

3.1 Oxidation of dextran with periodate

The aldehyde introduction in dextran by periodate was evaluated by iodometry, and the results are shown in Figure 2. The extent of oxidation was plotted against the weight ratio of periodate/dextran (the upper axis) and the molar ratio of periodate/glucose units (the lower axis) in the reaction, respectively. An almost linear increase was observed with the increase in the periodate concentration, and this result was in good agreement with those reported by other researchers.[33] A small amount of aldehyde was detected even when no periodate was added, which could be ascribed to reducing the terminal in intact dextran molecules. As is shown by the broken line, without this terminal aldehyde group, periodate effectively and stoichiometrically oxidized the glucose residues within an 1 h.

3.2 Acylation of ε-PL by anhydrides

To change the adhesive properties, some amino groups in ε-PL were acylated with acetic or succinic anhydride. The number of amino groups remaining was plotted against the concentration of anhydrides (the upper axis) and the molar ratio of anhydrides to amino groups in the reaction (the lower axis) is shown in Figure 3. In the case of acetic anhydride (Fig. 3A), the amino groups in ε-PL linearly decreased with its concentrations in the reaction. In contrast, the acylation was slightly suppressed when succinic anhydride was used (Fig. 3B). It is likely that the carboxyl groups introduced by succinic anhydrides enhanced the dissociation of the remaining amino groups that were in close proximity, and further acylation could be decelerated. Figure 4A and 4B show the aliphatic region of ^{1}H-NMR spectra of acylated ε-PL with acetic anhydride and succinic anhydride with various ratio against NH_2 groups, respectively. These charts also show that the acylation ratios determined by the ninhydrin assay are consistent with the ratios obtained from ^{1}H-NMR, and that the values are almost proportionate to the addition of anhydrides.

3.3 The gelation time of aldehyde dextran with ε-PL solution

In this experiment, 20 w/w% of aqueous aldehyde dextran with different extents of oxidation and 10 w/w% of ε-PL solution acylated with different amounts of anhydrides, were mixed (0.5 mL + 0.5 mL) and their gelation times were evaluated at 37°C. The results are given in Figure 5. The gelation times were plotted against the extents of oxidation in dextran (A), where 10 w/w% of ε-PL containing 2 w/w% of acetic anhydride was used. The gelation time markedly depended on the extent of

oxidation in dextran, which was easily predicted, and a wide range of gelation times from 3 (40% oxidation) to 60 sec (12%) was observed. When the oxidation extent was lower than 12%, such as 3.1 and 7.4% (shown in Figure 2), the time was longer than 300 sec.

The effect of acylation in ε-PL on the gelation time was measured when the dextran with 22.6% oxidation was used, and the results appear in Figure 5(B). Two different anhydrides, acetic and succinic anhydride, were compared. The time was increased with an increase in the anhydride concentration, and a time longer than 300 sec was observed regardless of the anhydride species when the concentration was higher than 3.5%. This result was due to the decrease in amino groups in ε-PL and enhancement of their dissociation by the production of acetic acid or acylated succinic acid. Although the degree of acylation promoted by acetic anhydrides was slightly higher than that by succinic anhydrides, as shown in Figure 3, its gelation time was shorter compared with succinic anhydrides at all of the concentrations of anhydride tested. This could have been caused by the dissociation of amino groups in ε-PL near the acylated succinic acids, and the effective suppression of the crosslinking reaction.

3.4 Bonding strength evaluation

The bonding strength of the dextran-PL adhesive was measured as the tensile-shearing strength of collagen casing sheets. Here, one typical composition of dextran-PL was selected, consisting of 20 w/w% of aqueous aldehyde dextran (22.6% oxidation) and 10 w/w% ε-PL containing 2 w/w% of acetic anhydride. The results are given in Table 1, with reference data for fibrin glue (Bolheal^{TM}). The

bonding strength of the fibrin glue to wet and swollen collagen sheets was as low as 14.1 gf in the average of five samples, and its low adhesion strength to wet organs has already been reported.[14,15] In contrast, dextran-PL gave 10 times higher strength than fibrin glue (185.9 gf, $p<0.001$), which suggested that dextran-PL was suitable for a wet surface, like soft tissues in applications. One reasonable explanation for the difference in bonding strength was the difference in the gelation times. Figure 5 shows that the gelation time of the dextran-PL used here was around 10 sec, which could allow the adhesive to spread sufficiently on the collagen sheets. In contrast, that of fibrin glue was too short for the test specimens, leading to data scattering. It is unlikely, however, that aldehyde dextran reacted with collagen molecules during the measurement, as will be described later.

3.5 Cytotoxicity testing

The cytotoxicity of aldehyde dextran and ε-PL to L929 cells was evaluated by the MTT assay. The IC50 of aldehyde dextran was plotted against the periodate concentrations during oxidation, and the results are given in Figure 6(A). With the increase in the periodate concentration, the IC50 decreased, which was caused by the introduction of aldehyde during the oxidation, but this did not show a linear profile as was predicted from Figure 2. Even at the highest concentration of periodate, the IC50 was higher than 5,000 ppm (0.5 w/w%) demonstrating low cytotoxicity.

The effects of acylation in ε-PL on the cytotoxicity are shown in Figure 6(B) as a function of the anhydride concentration during the acylation. In the case of acetic anhydride (triangles), the IC50 gradually decreases with the increase in its concentration up to 2,500 ppm. In contrast, the acylation

with succinic anhydrides (circles) showed lower cytotoxicity in the all range of the concentration compared with the acetic anhydrides. The one reason might be that the cation density was reduced by the introduction of carboxyl groups with the succinic anhydride treatment.

The cytotoxicity of other reference materials was also measured, and the results are summarized in Table 2, which also includes the results for typical aldehyde dextran and ε-PL. The IC50 of ε-PL was 1/200 of that of α-PL and 1/1,600 of that of poly(allylamine). Because ε-PL also has primary amino groups in its repeating units, like α-PL and poly(allylamine) (Figure 7), the differences in their cytotoxicity are worth noting. The IC50 of aldehyde dextran was also very low, at less than 1/1,700 the level of EDC and 1/3,400 the level of glutaraldehyde. The high toxicity of glutaraldehyde[39,40] and EDC[41] have already been reported, and the presence of such crosslinkers after the reaction has been a continuing problem associated with the biomedical applications of these compounds.

3.6 Reactivity of the aldehyde in aldehyde dextran and the amino groups in ε-PL

To obtain deeper insights into the low cytotoxicity of aldehyde dextran, the reactivity of aldehydes in dextran was compared with that in glutaraldehyde in PBS, where gelatin and albumin were chosen as substrate amino species. The results are shown in Figure 8(A). The amino group consumption in gelatin (open marks) and albumin (solid marks) by aldehydes was plotted against the molar concentration of aldehyde in the reaction. In the case of aldehyde dextran (triangles), only slight amounts of amino group consumption were observed at the concentrations lower than 30 mM, regardless of the protein species, and there was a gradual consumption with increasing concentrations.

In contrast, the consumption of amino groups was higher than 80% for gelatin and 70% for albumin even at the lowest aldehyde concentration (15.5 mM) in this experiment when glutaraldehyde was used (circles). These findings indicated that the aldehydes in the dextran have low reactivity, which might have been responsible for the low cytotoxicity of aldehyde dextran.

The reactivity of amino groups in ε-PL was also compared with simple amino species such as glycine, L-lysine, and gelatin (Figure 8(B)). To ensure that the reaction pH remained constant (pH=7.4), 0.3 M of phosphate buffer was used instead of PBS. Here, the ratio of the molar concentration of aldehydes in dextran to amino groups in the reaction varied from 1 to 50. Although the degree of amino groups consumed increased with the increase in the aldehyde ratio regardless of amine species, that of ε-PL was far higher than those of other amines over the range of the concentrations.

4. Discussion

We synthesized a dextran-based adhesive which easily formed a hydrogel without any low-molecular weight or cytotoxic crosslinkers, and a combination of aldehyde dextran with ε-PL provided a variety of useful physicochemical properties. Both the oxidation of dextran with periodate and acylation of ε-PL with anhydrides proceeded almost stoichiometrically under mild conditions, as shown in Figures 2 and 3. Changing the extents of oxidation in dextran and acylation in the ε-PL provided different gelation times, even though the same concentrations of solutions were used. Without the need for pH adjustment by buffer molecules[42] the gelation time could be easily controlled, leading to a variety of applications.

Our dextran-based adhesive could be typically characterized by its low cytotoxicity. Although they have excellent physical properties, it is difficult to utilize simple aldehyde species like formaldehyde and glutaraldehyde, or other crosslinkers such as carbodiimides, for biomaterial applications because of their high cytotoxicity, as indicated in Table 2. The high cytotoxicity of EDC and glutaraldehyde can be ascribed to their reactions with organs or cells in the body. Although both glutaraldehyde and aldehyde dextran are aldehyde species, the unique structural features of the aldehyde dextran led to its much lower IC50 value, as determined in section 2.1, and shown in the right column in the Table 2. Although the cytotoxicity of aldehyde dextran was enhanced by the oxidation and introduction of aldehyde (Figure 6), the cytotoxicity was still 1/1,000 that of glutaraldehyde and EDC. Even if the IC50 was expressed based on the concentration of aldehyde groups, the cytotoxicity of aldehyde dextran was far lower than that of glutaraldehyde. Ito et al. previously pointed out the low cytotoxicity of aldehyde groups introduced in polysaccharides like hyaluronic acid.[43] The reactivity of aldehyde dextran, was therefore compared with that of glutaraldehyde. At the same molar concentration, the reactivity of the aldehyde groups in dextran was lower than that of glutaraldehyde when gelatin and albumin were chosen as the reaction substrates, as shown in Figure 8(A). The dextran structure following periodate oxidation was already investigated, and no aldehyde peak in the IR spectrum was observed.[44,45] Furthermore, ald-enol transition formation of aldehydes in dextran was proposed by the results of the ^{1}H- and ^{13}C-NMR analyses.[46] These reports might suggest that aldehydes introduced in polysaccharides by periodate are rather stable and show low reactivity with amines. They might therefore be inherently different from simple aldehydes like glutaraldehyde, which is one possible

explanation for the low cytotoxicity of aldehyde dextran. The low reactivity and cytotoxicity might be also related to the high molecular weight of aldehyde dextran and the steric effect arising from the high molecular weight. Even if only a few aldehyde groups on oxidized dextran were reacted with amino groups, the oxidized dextran macromolecules cannot move freely or leach out from the crosslinked network, and the steric hindrance of the polymer may also slow down the reaction between unreacted aldehyde groups with surrounding cells. But for small molecules like glutaraldehyde, the unreacted molecules can easily leach out from the crosslinked network and react with surrounding cells and tissues.

In contrast, the amino groups in ε-PL were highly reactive at pH 7.4 compared with those in glycine, L-lysine, and gelatin at the same molar concentrations of amino groups, as shown in Figure 8(B). The differences in their reactivity could be explained by the extent of dissociation of the amino groups at pH 7.4. The pKb values of the amino groups in glycine and L-lysine are 4.22, 4.82 (alpha amino) and 3.21 (epsilon amino group), which indicates that almost all of the amino groups are dissociated at neutral pH. It is likely that the amino groups in gelatin assigned to such as lysine and arginine residues are also most dissociated. On the other hand, the dissociation of the alpha amino groups in ε-PL is thought to be considerably suppressed by the adjacent carbonyl groups in the repeating amide bonds (Figure 1). Therefore, the reactivity of amino groups in ε-PL is far higher than that of other amino species at pH 7.4. This poor dissociation of the amino groups in ε-PL could lead to its far lower cytotoxicity compared with α-PL and poly(allylamine), as is shown in Table 2. Indeed, the cytotoxicity of poly(allylamine) was as high as that of glutaraldehyde, one of highly cytotoxic

crosslinkers. Based on their chemical structures, the dissociation of amino groups occurred easily in aqueous media at neutral pH for both molecules. In contrast, the alpha amino groups in ε-PL were adjacent to carbonyl groups in amide bond repeats (as shown in Figure 1), which could considerably suppress the dissociation of the amino groups. Therefore, it is likely that the difficulty in cationization of ε-PL was associated with its low cytotoxicity. At neutral pH (7.4), most of amino groups on ε-PL are not dissociated, and thus they may not strongly interact with negative cell membrane. Many kinds of cationic polymers, such as poly(ethyleneimine) and α-PL have been used for gene delivery,[47-49] and their cytotoxicity (as well as their efficacy) has been discussed, which is mainly determined by their cationic nature.[50] In other words, ε-PL is not a candidate polymer for gene delivery because of its poor cationic nature, though the high reactivity of amino groups is good for the crosslinking reaction with the poorly reactive aldehydes in dextran.

From the results obtained in Figures 6 (A) and (B), the IC50 values of aldehyde dextran and acylated ε-PL were determined to be higher than 5,000 ppm, suggesting that they have low cytotoxicity. Especially from the Figure 6(B), the IC50 of acetic anhydride reacted ε-PL gradually decreases with the increase in its concentration up to 2,500 ppm. Because acetic anhydrides reacted with amino groups and left free acetic acids after the acylation, it is likely that acetic acid enhanced the cytotoxicity of ε-PL, whose IC50 was 700 ppm. In contrast, no free acids were left by the acylation with succinic anhydrides, leading to the lower cytotoxicity compared with acetic anhydride. As no gelation was observed when the anhydride concentration was 4 w/w%, regardless of the anhydride species (as shown in Figure 5B), the IC50 of ε-PL crosslinkable with aldehyde dextran was higher

than 5,000 ppm, which is far lower than that of succinic acid (IC50 = 1,000 ppm), hydrolization products of succinic anhydrides and various kinds of food additives examined for reference.

In conclusion, we successfully synthesized low cytotoxic dextran-based tissue adhesive which easily formed a hydrogel without cytotoxic crosslinkers, The low cytotoxicity of aldehyde in the oxidized dextran was likely to ascribed to its low reactivity with amine species due to the ald-enol transition. And high reactivity of the α-amino groups in ε-PL could be ascribed to their low dissociation at neutral condition, which leads to low cytotoxicity. We could easily control the gelation time by the extent of oxidation in dextran and of acylation in ε-PL. The hydeogel of the aldehyde-dextran and ε-PL showed good adhesive strength against collagen sheets as a tissue adhesive. Therefore, we concluded that this formulation for the preparation of hydrogel can be useful to design novel tissue adhesives with low cytotoxicity.

Disclosures

The authors have no conflicts of interest to declare.

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Table 1. Shearing bonding strength of dextran-PL and fibrin glue to swollen collagen seats at 25°C.

adhesive	shearing bonding strength / gf					average±s.d. /gf
dextran-PL	185.2	173.0	199.0	196.9	175.3	185.9±10.7***
fibrin glue	10.2	25.8	14.3	10.7	9.4	14.1± 6.1

Different from fibrin glue at *** $p<0.001$.

Table 2. Cytotoxicity of aldehyde dextran, ε-PL, and other reference materials to L929 cells.

material	IC50[3)]	
	ppm	-CHO / 10^{-6}M
α-PL	34.1 ± 0.6	———
poly(allylamine)	5.0 ± 0.1	———
ε-PL[1)]	8,00 ± 80***	———
EDC	5.6 ± 0.1	———
glutaraldehyde	2.9 ± 0.1	89.1 3.1
	10,000 ± 100###	28,400 ± 100†††

1) acetic anhydride concentration was 2 w/w% in acylation.
2) 0.25 g of periodate was added to 1 g of dextran in oxidation (22.6% oxidation).
3) data = average±s.d.(n=8x8).

Different from alpha-PL and poly(allylamine) at *** $p<0.001$; different from EDC and glutaraldehyde at ### $p<0.001$; different from glutaraldehyde at ††† $p<0.001$.

Figures

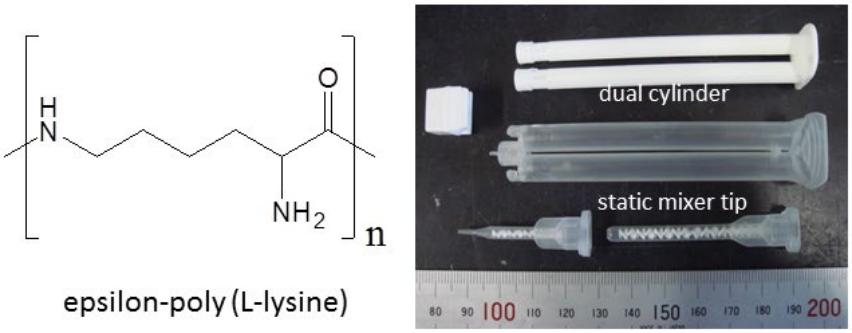

Fig.1

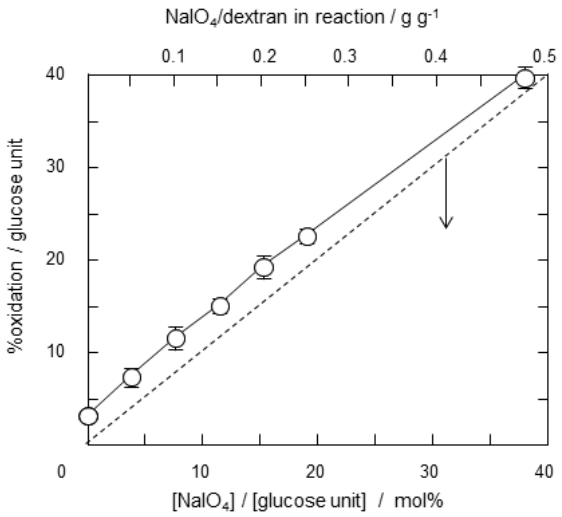

Fig.2

Figure 2. The effects of the periodate concentrations on the oxidation of dextran. A 20g sample of dextran in 80 mL of water and 0-10g of sodium periodate in 40 mL of water were mixed, and the reaction was allowed to proceed at 50ºC for 1h.

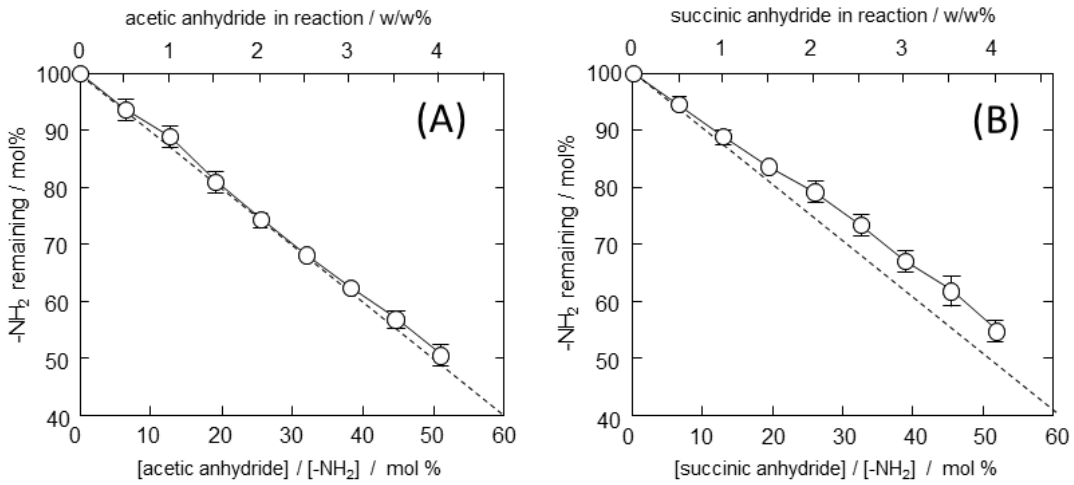

Fig.3

Figure 3. The effects of the anhydride concentrations on the acylation of ε-PL. A 10 w/w% of ε-PL was reacted with 0-4 w/w% of acetic (A) and succinic anhydride (B) at 50°C for 1h.

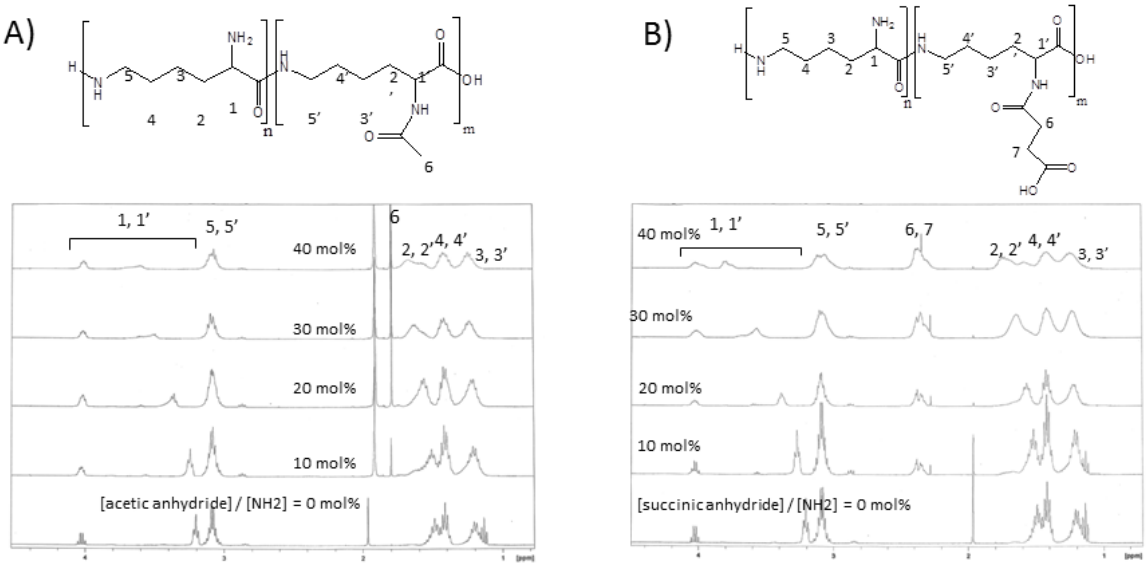

Fig4

Figure 4. ^{1}H-NMR spectra of acylated ε-PL with (A) acetic anhydride and (B) succinic anhydride.

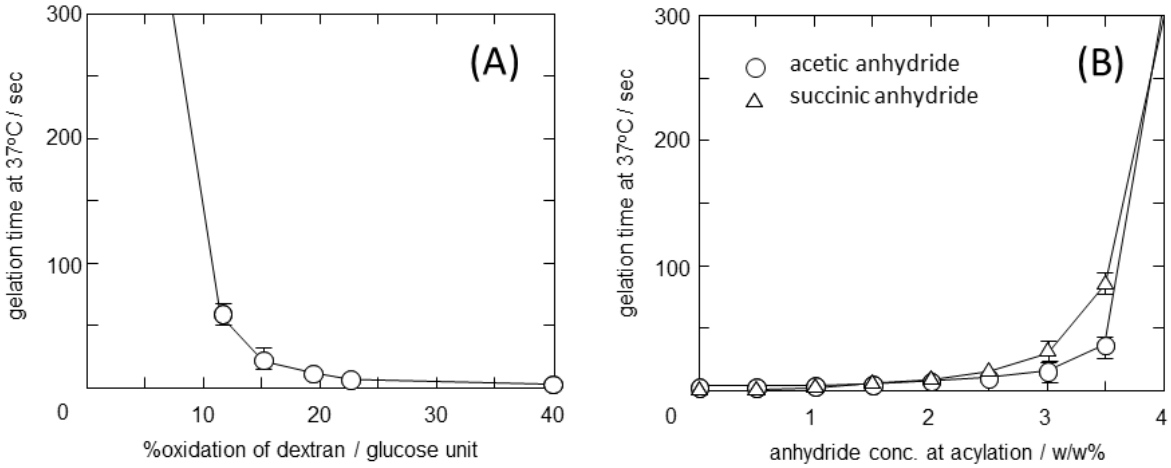

Fig.5

Figure 5. The gelation time is dependent on the extent of (A) oxidation in dextran and (B) the anhydride concentrations in ε-PL acylation. (A) A 0.5 mL aliquot of 20 w/w% of aqueous aldehyde dextran with different extents of oxidation and 0.5 mL of 10 w/w% ε-PL containing 2 w/w% of acetic anhydride were mixed at 37ºC. (B) 0.5 mL of 20 w/w% of aqueous aldehyde with 22.6% oxidation and 0.5 mL of 10 w/w% ε-PL containing different concentrations of acetic or succinic anhydride were mixed.

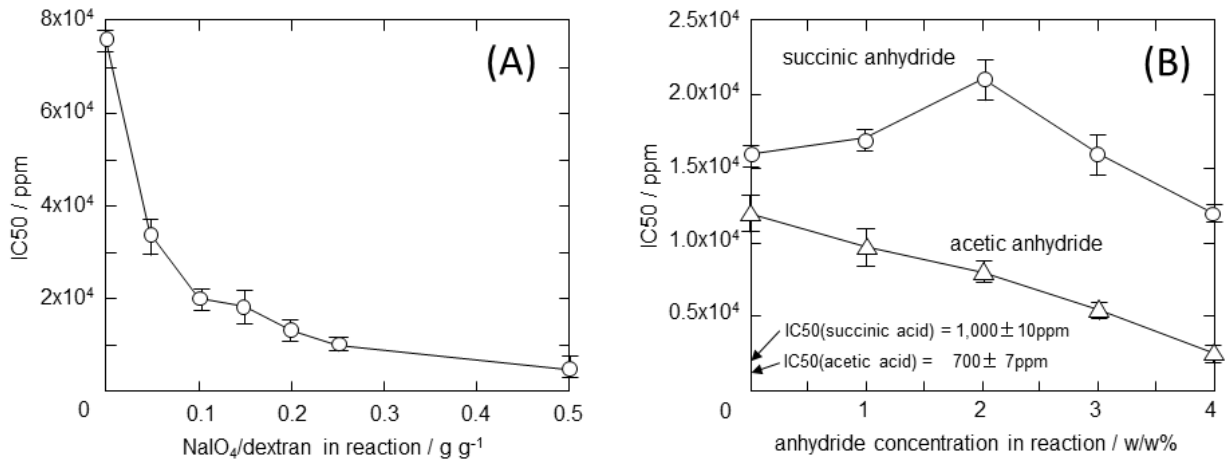

Fig.6

Figure 6. The cytotoxicity of (A) aldehyde dextran and (B) ε-PL acylated with acetic and succinic anhydride to mouse L929 cells. The IC50 indicates the concentration of substance in culture which reduces cell viability by 50%.

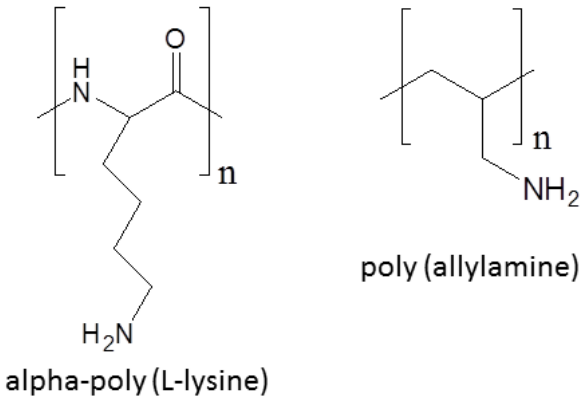

Fig.7

Figure7. The chemical structures of alpha-poly(L-lysine) and poly(allylamine).

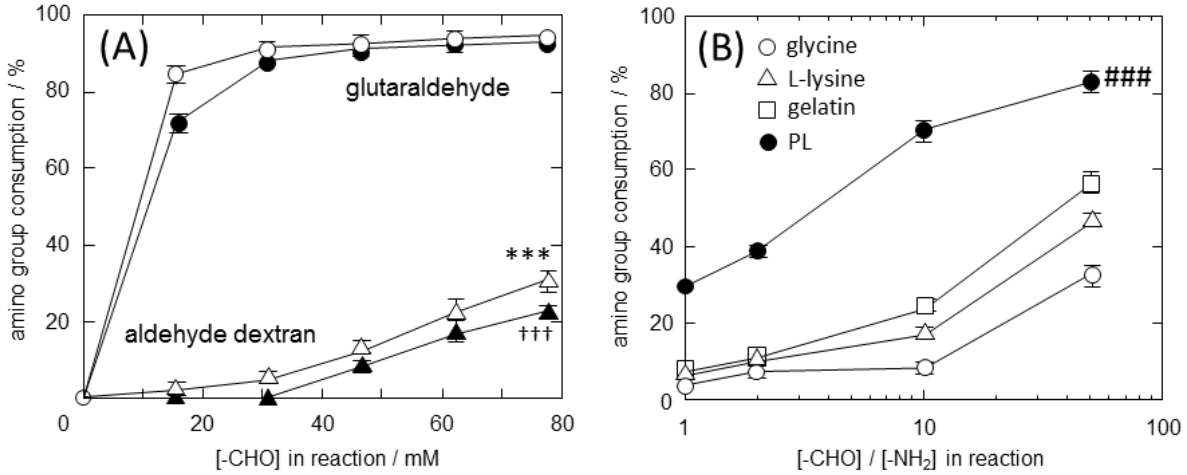

Fig.8

Figure 8. The reactivity of aldehydes in dextran and the amino grops in ε-PL. (A) A comparison of the reactivity of aldehyde groups in aldehyde dextran and glutaraldehyde with gelatin (open) and albumin (solid marks) at 37ºC in PBS. (B) A comparison of the amino group reactivity with aldehyde dextran at 37ºC in phosphate buffer (pH 7.4). Different from glutaraldehyde with gelatin at *** $p<0.001$; different from glutaraldehyde with albumin at †††$p<0.001$; different from glycine, L-lysine and gelatin at ### $p<0.001$.