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Feasibility study of a novel crosslinking reagent (alginate dialdehyde) for biological tissue fixation

Yuanting Xu[a], Li Li[b], Xixun Yu[a,*], Zhipeng Gu[a], Xu Zhang[a]

[a] College of Polymer Science and Engineering, Sichuan University, Chengdu, Sichuan Province 610065, PR China
[b] Department of Oncology, the 452 Hospital of Chinese PLA, Chengdu, Sichuan Province 610021, PR China

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ABSTRACT

Biological tissues must be chemically fixed before they can be implanted in humans. To overcome the cytotoxicity of the current chemical reagents used to fix bioprostheses, a naturally occurring crosslinking agent, alginate dialdehyde (ADA), was employed to fix biological tissues in this feasibility study. In this work, the crosslinking characteristics and the cytotoxicity of ADA-fixed biological tissues were investigated. The results indicated that ADA-fixed tissues are in possession of the fixation index and mechanical strength comparable to glutaraldehyde-fixed counterparts and superior to polyepoxy-fixed counterparts. The histological examination confirmed that the natural structure of the tissues preserved well after ADA fixation. Moreover, the results obtained in the MTT study further indicated that the cytotoxicity of ADA-fixed tissues was significantly lower than that of glutaraldehyde-fixed and polyepoxy-fixed tissues. In conclusion, the results of this vitro study demonstrate that ADA is an effective agent in the fixation of biological tissue.

1. Introduction

Biological tissues have been used extensively to fabricate various bioprostheses such as heart valves (Zhai et al., 2006), vascular grafts (Yu, Liu, Xu, & Wan, 2010) and ligament substitutes (Sung, Shih, & Hsu, 1996). However, it is known that these biological tissues must be chemically fixed before they can be implanted in humans due to the immediate degradation and the presence of antigenicity of naturally derived tissues. Although various synthetic crosslinking reagents, such as formaldehyde, glutaraldehyde (Cheung, Perelman, Ko, & Nimni, 1985), and polyepoxy compound (Sung, Hsu, Shih, & Lin, 1996; Sung, Shih, & Hsu, 1996), have been widely used in the pretreatment of natural biological tissues, these synthetic crosslinking reagents are all highly (or relatively highly) cytotoxic (Nishi, Nakajima, & Ikada, 1995; Yu, Wan, & Chen, 2008). It is therefore desirable to provide a novel crosslinking agent that endows bioprostheses with high fixation index, good mechanical properties and low cytotoxicity.

Alginate (ALG), derived from brown algae, is an important naturally occurring polysaccharide for widespread biomedical applications (Isiklan, Inal, Kursun, & Ercan, 2010; Li, Ramay, Hauch, Xiao, & Zhang, 2005; Ramesh Babu, Sairam, Hosamani, & Aminabhavi, 2007; Yang, Xie, & He, 2010), mainly due to its high biocompatibility. It was reported that alginate can be oxidized with periodate and form multiple functional aldehyde groups (alginate dialdehyde, ADA) (Bouhadir et al., 2001). Therefore, ADA may react with the free amino groups within biological tissues using its aldehyde functional group in the same way as glutaraldehyde. The aforementioned results prompted us to evaluate the feasibility of using ADA as a novel crosslinking reagent in the fixation of biological tissues.

Attempting to achieve this goal, we prepared the ADA reagent under controlled conditions according to the reported method (Laurienzo, Malinconico, Motta, & Vicinanza, 2005; Vieira, Cestari, Airoldi, & Loh, 2008). And then the chemical structure and oxidized degree of ADA were investigated. Subsequently, the ADA reagent was used to fix porcine aortas. The crosslinking characteristics and cytotoxicity of ADA-fixed biological tissues were evaluated. In the study, fresh porcine aortas procured from a slaughterhouse were used as raw materials. Glutaraldehyde (GA) and polyepoxy compound (PC) that have been used extensively in chemically modifying biological tissues were used as controls. The chemical structure of these crosslinking reagents is shown in Fig. 1.

2. Materials and methods

2.1. Materials

Sodium alginate (viscosity: 495 cps at 25 °C) was obtained from Qingdao Jingyan Biotechnology Co. Ltd (China). Glutaraldehyde, polyepoxy compound and diphenyl tetrazolium bromide (MTT)

* Corresponding author at: School of Polymer Science and Engineering, Sichuan University, Chengdu, Sichuan Province 610065, PR China. Tel.: +86 28 85405404; fax: +86 28 85405402.
E-mail address: yuxixun@163.com (X. Yu).

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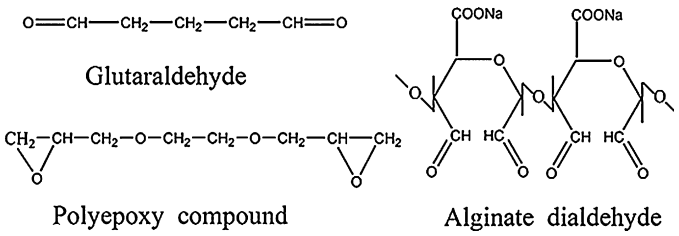
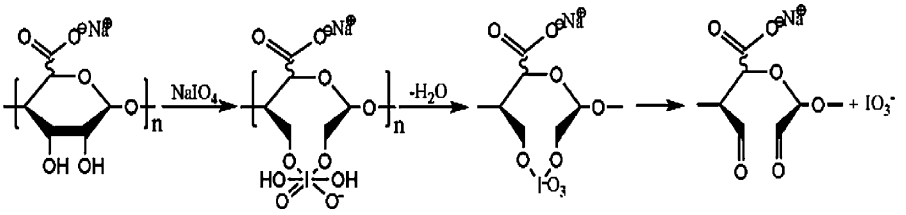


Fig. 1. The chemical structure of glutaraldehyde, polyepoxy compound and alginate dialdehyde in the study.

were obtained from Sigma–Aldrich (St. Louis, MO, USA). Triton X-100 was obtained from Amresco Co. (USA). DNaseI and RNseA were obtained from Aladdin Co (Shanghai, China). Phosphate buffered saline (PBS, pH 7.4, 0.01 mol/L) was prepared by dissolving 1.145 g of disodium hydrogen phosphate dodecahydrate, 0.2 g of potassium dihydrogen phosphate, 0.2 g of potassium chloride and 8 g of sodium chloride in 1 L distilled water. Sodium carbonate/sodium bicarbonate (pH 10.5, 0.21 M/0.02 M) was prepared by dissolving 22.26 g of sodium carbonate and 1.68 g of sodium bicarbonate in 1 L distilled water. Sodium periodate and other reagents (analytical grade or equivalent grade) were obtained from Kelong Co. (Chengdu, China). Distilled water was employed throughout.

2.2. Preparation and assessment of ADA

ADA was prepared as the method reported by Vieira et al. (2008) with slight modification for better oxidation. Briefly, the sodium alginate (5 g) was dissolved in 200 ml distilled water and 50 ml pure ethanol, and then were mixed with 5.7 g of sodium periodate and magnetic stirred in dark at room temperature to obtain the product. All the oxidation was followed as in Eq. (1) (Wang et al., 2010):



(1)

The reaction was neutralized after 24 h by 10 ml of ethylene glycol under dark for 2 h. The product was purified by precipitation with the addition of 5 g sodium chloride and 800 ml pure ethanol. The polymer was dissolved in about 100 ml distilled water again and re-precipitated by the addition of 600 ml ethanol. The procedure was repeated three times. Furthermore, the product solution was dialyzed using dialysis tube (MWCO, 3500) against distilled water with several changes of water until the dialyzate was periodate free (Balakrishnan, Lesieur, Labarre, & Jayakrishnan, 2005). The dialyzate was then lyophilized to obtain the product.

In the assessment studies, the chemical structure of ADA was characterized by FTIR. Dry samples were pressed as KBr pellets using a hydraulic press Carver. FTIR spectra of various samples were recorded as % transmittance using FTIR spectrometer (Nicolet 560, USA). The degree of oxidation was determined by potentiometric titration aldehyde groups by hydroxylamine hydrochloride/sodium hydroxide method (Zhao & Heindel, 1991). Then, the value of oxidation degree (OD) could be calculated by Eq. (2). The molecular weight of sodium alginate monomer is 198.11.

$$\mathrm{OD} = \frac{n(\mathrm{CHO})/2}{w_{\mathrm{alginate}}/198.11} \quad (2)$$

2.3. Decellularization and crosslinking process

In the study of the crosslinking characteristics of the ADA-fixed tissue, fresh porcine aortas procured from a local abattoir were used as raw materials. The procured aortas were brought to the laboratory in sterile phosphate buffered saline. Upon return, the excess blood on the tissues was immediately removed by rinsed with fresh saline and the adherent fat was also carefully trimmed from the arotas surface with a scalpel. The maximum time period between retrieval and initiation of tissue decellularization was less than 6 h (Yu et al., 2010). Subsequently, the decellularization process was conducted according to previously reported methods (Liang, Chang, Hsu, Lee, & Sung, 2004). At first, the aortas were treated by 0.1% trypsin and 0.02% EDTA solution at 37 °C for 4 h, and then washed with sterile PBS, followed by treatment in a Tris solution with 1% Triton X-100 for 48 h. Finally, the tissues were further treated in RNaseA (0.02 mg/ml) and DNaseI (0.2 mg/ml) for 4 h with agitating at 37 °C to remove cellular components.

After washing with D-Hanks solution, the decellularized aortas were fixed in a 0.625% glutaraldehyde solution, a 4% polyepoxy compound solution, and 15% ADA solution at 37 °C for 5 days (Sung, Shih, et al., 1996). The polyepoxy compound solution was buffered with sodium carbonate/sodium bicarbonate while the glutaraldehyde and ADA solutions were buffered with phosphate buffered saline. The samples of each group were taken out at predetermined fixation periods (from 15 min to 120 h) and then the crosslinking characteristics of each group were determined respectively.

2.4. Fixation index determination

It is known that the amount of free amino groups in the tested tissue, after heating with ninhydrin (NHN), is proportional to the optical absorbance of the solution. So the ninhydrin assay was often used to determine the free-amino group content of each test sample. The fixation index (FI) was an indicator of the degree of crosslinking, and defined as Eq. (3) (Sung, Shih, et al., 1996):

$$\mathrm{FI} = \frac{(\mathrm{NHN\ reactive\ amine})\ \ \mathrm{fresh} - (\mathrm{NHN\ reactive\ amine})\ \ \mathrm{fixed}}{(\mathrm{NHN\ reactive\ amine})\ \ \mathrm{fresh}} \quad (3)$$

The tested sample was first lyophilized for 24 h and then weighed. Subsequently, the lyophilized tissue was heated with an NHN solution to boiling for 20 min, and then the optical absorbance of the solution was recorded by a spectrophotometer (UV-752, Shanghai). Glycine at various known concentrations was used as standard.

2.5. Mechanical testing

The mechanical properties of the studied tissues were examined based on our previous described methods (Yu et al., 2010). To prepare specimens for tensile strength testing, each sample from individual group was cut along the collagen fiber direction to yield one strip 4 mm × 40 mm, then the thickness and width of the sample was obtained using a micrometer. Five tissue straps in each group were tested on an Instron material testing machine (Instron Co., USA) at a constant speed of 10 mm/min. After measurement, the

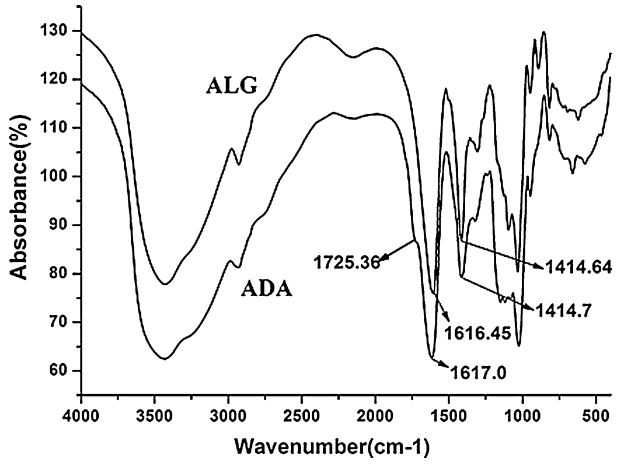


Fig. 2. FTIR spectrum of the ALG and ADA. The major absorbance for the functional groups are noted.

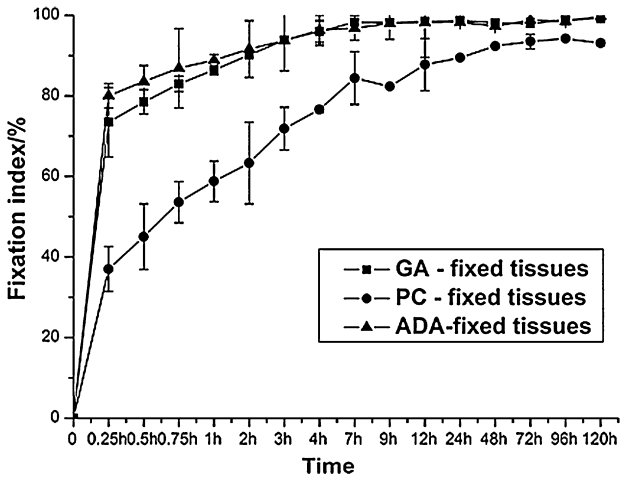


Fig. 3. Fixation index of the tissues fixed with GA, PC and ADA.

ultimate tensile strain and the ultimate tensile stress were recorded before failure. The ultimate elastic modulus was determined from the stress–strain curves. During testing, the tissue strips were kept in air.

2.6. Morphologic observation

The decellularized and fixed aorta scaffolds were photographed using a digital camera. The specimens were fixed in 4% formaldehyde for 72 h. This was followed by hematoxylin & eosin (H&E) staining, to perform morphologic observation. Meanwhile, to observe the structure integrity of the tissues, the decellularized and crosslinked samples were also examined histologically by Masson staining for collagen fibers and Verhoeff iron hematoxylin staining for elastic fibers.

2.7. Cytoxicity study of fixed tissues

The cytotoxicity of fixed tissues was evaluated in vitro using a mouse-derived established cell line of L929 fibroblasts. The sterilized tissues were placed in the saline for 24 h at 37 °C in 5% CO_2 for extraction liquid. The cells were trypsinized and inoculated into 96-well plates at the density of 2×10^3 cells/well in 100 μl DMEM medium. Meanwhile, 100 μl extraction liquid of fixed tissues was added into each well. The cell culture was performed at 37 °C in humidified 95% air/5% CO_2. Using the MTT assay, the viable cells cultured in each well were determined at 1, 3, 5 and 7 days after cell seeding. Details of the methodology used in the MTT assay were previously described (Yu et al., 2008).

2.8. Statistical analysis

Statistical analysis was performed with SPSS (v13.0). Quantitative data are presented as a mean value with its standard deviation indicated (mean ± SD), and statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Preparation and assessment of ADA

The purified ADA product was a white powder and the FTIR spectrum of ALG and ADA were shown in Fig. 2. The spectrum of ALG showed the absorption bands at 1617 and 1415 cm^{-1} was assigned to asymmetric and symmetric stretching peaks of the carboxylate salt groups. While in the spectrum of ADA, the absence of a band at 1725 cm^{-1} clearly indicated the formation of the Schiff's base involving the free aldehyde groups of the alginate dialdehyde. As a result, the presence alginate bands in the FTIR spectrum of ADA confirmed the successful preparation of the product (Vieira et al., 2008). Meanwhile, the degree of oxidation, defined as the percentage of oxidized uronic acid units in the ADA, was determined to be 47.96 ± 2.67% as measured by the hydroxylamine hydrochloride method.

3.2. Decellularization and crosslinking of the tissues

The fresh, glutaraldehyde-fixed, polyepoxy-fixed and ADA-fixed tissues were photographed (the photograph was not shown in this paper). It was found that the polyepoxy-fixed tissue was white as same as the fresh one, whereas the color of both glutaraldehyde-fixed and ADA-fixed tissue was turned yellow. It was due to the different crosslinking ways between aldehyde groups and epoxide groups. The polyepoxy compound fixed the biological tissues with its epoxide groups, whereas the ADA and glutaraldehyde fixed tissues with their aldehyde groups.

3.3. Fixation index determination

The fixation index is used to estimate the amount of free amino groups left in the biological tissues subsequent to the fixation and the crosslinking degree of the fixed biological tissues. A higher fixation index often implies a lower level of free amino groups in the fixed biological tissues and a higher crosslinking degree in the fixed tissues (Shen et al., 1994). As shown in Fig. 3, the fixation index of the GA, and ADA-fixed tissues increased more rapidly than its PC-fixed counterpart, which may be due to the slower reaction rate of epoxide groups with collagen as compared to that of aldehyde groups. The similar finding was also reported by Sung et al. (Sung, Shih, et al., 1996). In addition, the crosslinking rate of the ADA-fixed tissue was a little faster than that of the GA-fixed tissue at the beginning of fixation, which may be a result of higher concentration of aldehyde groups. After 7 h of fixation, the fixation indices of the GA and ADA-fixed tissues nearly reached maximum (>90%). However, it was noticed that a longer fixation time was required to produce a similar crosslinking degree for the polyepoxy fixation. This suggested the 15% ADA initial fixative concentrations showed the outstanding fixative efficiency, the maximum (>90%) of fixation index could be achieved in 7 h. In a word, the fixation index of the ADA was comparable as GA and superior to PC.

Table 1
Mechanical properties of the fresh and fixed tissues ($n = 5$).

Treated samples	Ultimate tensile strain (%)	Ultimate tensile stress (MPa)	E-modulus (MPa)
Fresh	125.06 ± 18.04[#]	1.24 ± 0.23	2.45 ± 1.04
GA-fixed	86.48 ± 10.52	1.38 ± 0.82	2.75 ± 1.86
PC-fixed	81.12 ± 11.53	0.59 ± 0.32[*]	1.24 ± 0.48
ADA-fixed	92.72 ± 6.59	1.29 ± 0.52	2.84 ± 1.27

[*] $P < 0.05$ compared with fresh tissues.
[#] $P < 0.05$ compared with GA, PC and ADA-fixed tissues.

3.4. Mechanical testing

Crosslinking of a biological tissue by a crosslinking reagent may increase its mechanical strength. The values of "ultimate tensile strain", "ultimate tensile stress" and "e-modulus" of the tested samples were presented in Table 1. As we can see, the fresh samples were in possession of the greatest ultimate tensile strain which means the good elasticity. In contrast, it was found that the ultimate tensile stress and e-modulus of the ADA- and GA-fixed tissues were significant higher than the fresh tissues, while the polyepoxy-fixed samples were the lowest. Based on these values, we can conclude that of the fixed tissues, the mechanical strength of the polyepoxy-fixed tissue was the lowest while that of the ADA-fixed tissue was comparable to the glutaraldehyde-fixed tissue. This result is also in agreement with the result of fixation index.

Glutaraldehyde and polyepoxy compound are two typical crosslinking reagents which have been used extensively in chemically modifying biological tissues. As shown in Fig. 1, chemical structures of ADA and glutaraldehyde are similar. It was speculated that ADA could react with the free amino groups within biological tissues using its aldehyde functional group in the same way as glutaraldehyde, which result to the similar crosslinking properties of glutaraldehyde-fixed and ADA-fixed tissues. As for the relatively poor property of polyepoxy-fixed tissues, this is mainly due to the different crosslinking mechanism. The glutaraldehyde and ADA use its aldehyde functional groups to crosslink collagen fibers. The network crosslinking structure is created intramolecularly and intermolecularly within collagen fibers. However, the epoxy compound utilizes its epoxide functional groups to crosslink collagen fibers. In this fashion, a linear crosslinking structure between adjacent tropocollagen molecules may be produced intermolecularly (Sung, Chang, Chiu, Chen, & Liang, 1999).

Additionally, in the fixation of biological tissues, crosslinking means that more than one functional groups in the fixative react with the amino groups and form a bridge within the tissue. However, if only one functional group reacts, it leads only to capping of the reacted amino groups without forming a bridge within the tissue. This phenomenon is called masking (Sung, Shih, et al., 1996). The mechanical testing of the fixation of biological tissues is a means to identify the effective crosslinking and the masking of the free amino group in the fixed biological tissues.

It was obvious that the ADA-fixed tissues presented significant increase in mechanical strength. This result indicated that the tissues were crosslinked effectively by ADA and the masking of the free amino group in tissues did not appear in this fixation processing.

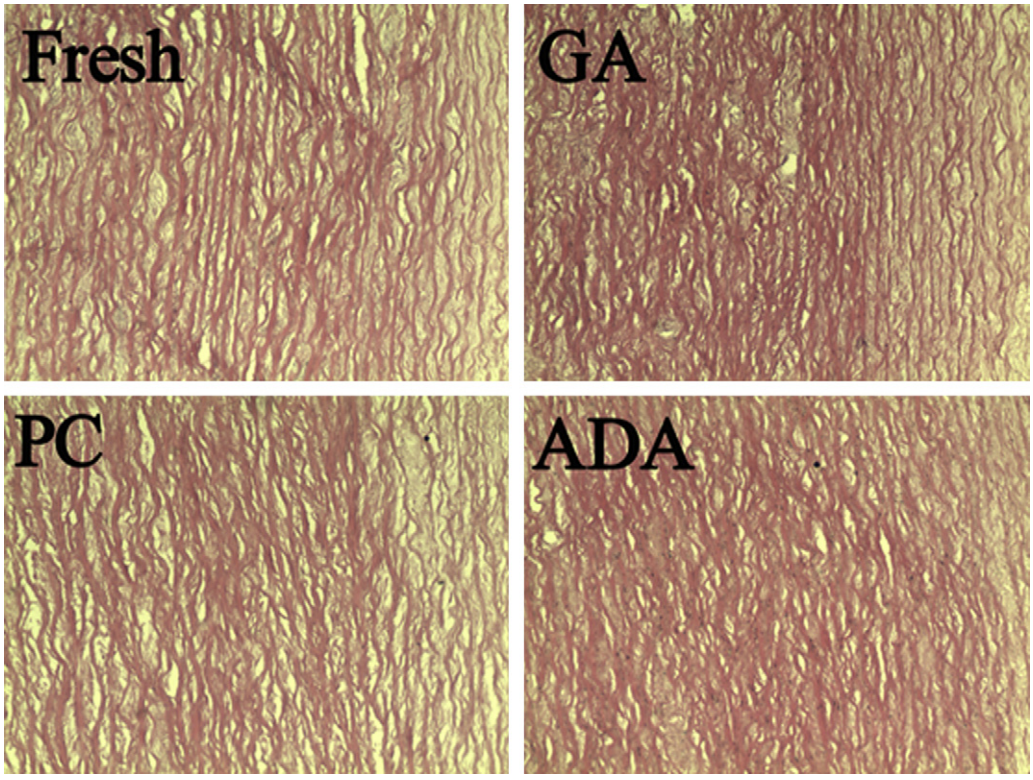


Fig. 4. Photomicrographs (hematoxylin and eosin staining, 200× magnification) of the fresh tissue, GA-fixed tissue, PC-fixed tissue, and ADA-fixed tissue.

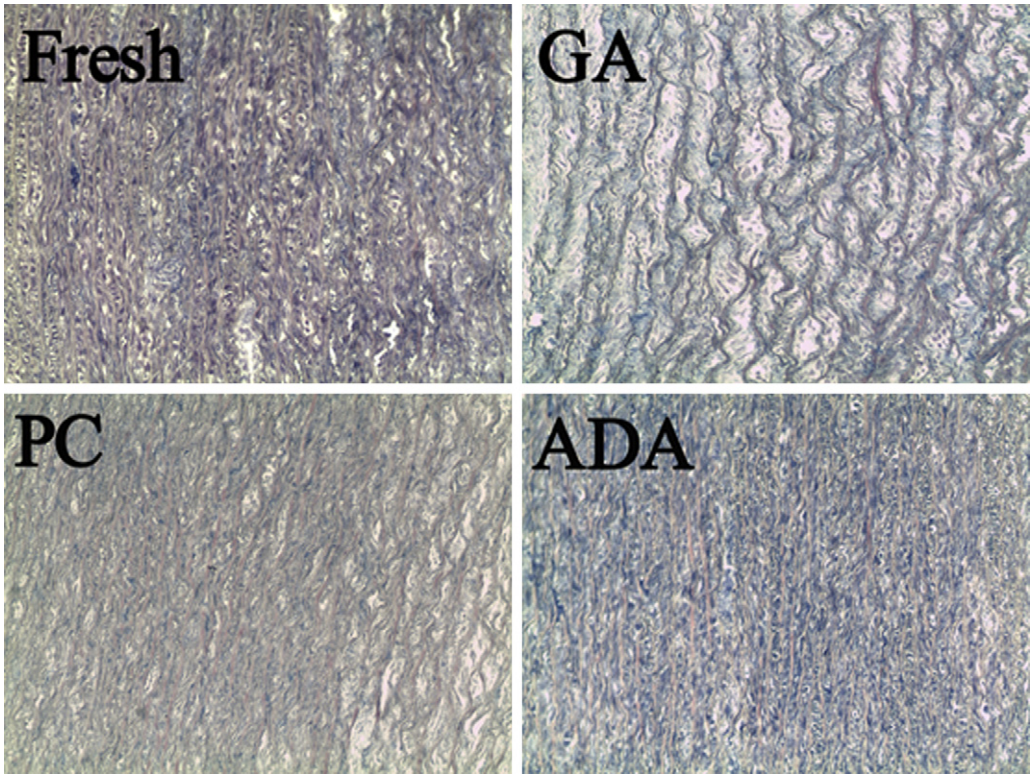


Fig. 5. Photomicrographs (Masson staining, 200× magnification) of the fresh tissue, GA-fixed tissue, PC-fixed tissue, and ADA-fixed tissue.

3.5. Morphologic observation

Histological examination mainly showed the total framework of the fixation of biological tissues. Fig. 4 presents the photomicrographs of the fresh, glutaraldehyde-fixed, polyepoxy-fixed, and ADA-fixed porcine aortas, stained with hematoxylin and eosin. It was noted that there was no any sign of remaining cellular components in the tissues, which indicated the successful

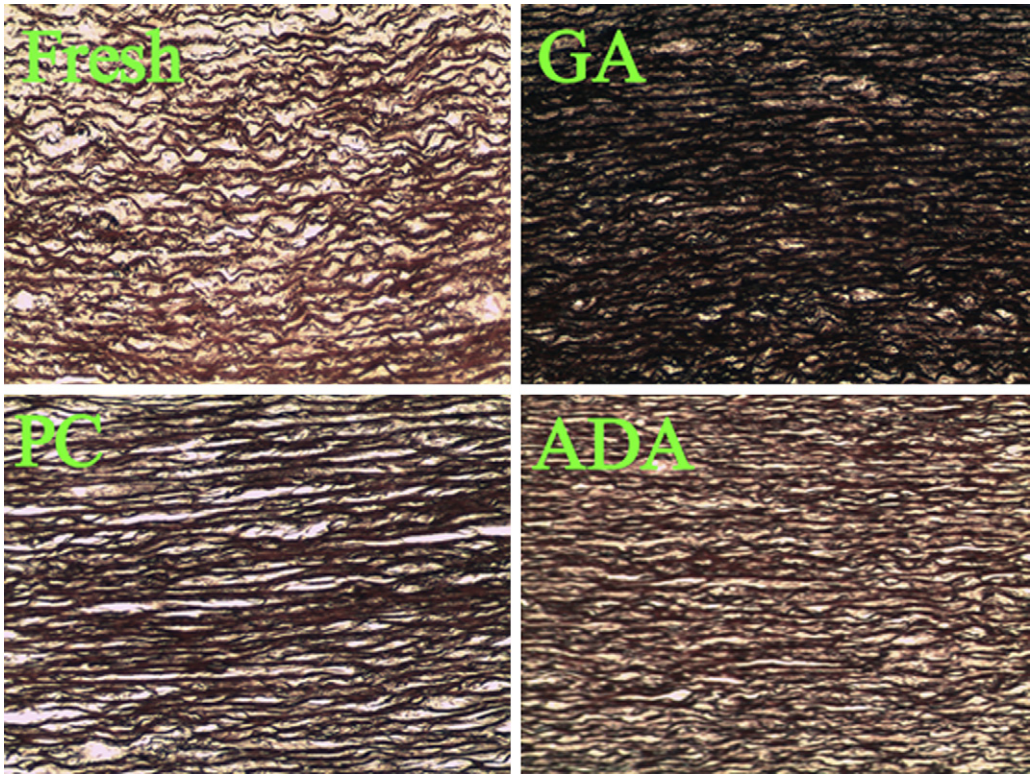


Fig. 6. Photomicrographs (Verhoeff iron hematoxylin staining,) of the fresh tissue, GA-fixed tissue, PC-fixed tissue, and ADA-fixed tissue.

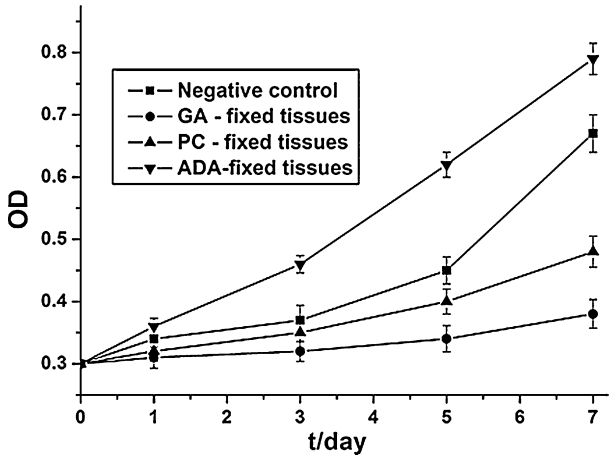


Fig. 7. Proliferation of the L929 fibroblasts cultured in the extraction liquid of various fixed tissues.

decellularization process. This complete removal of original resident cells in biological tissue scaffolds can reduce the antigenicity derived from cell, and furthermore diminished the immune response elicited to these materials in vivo (Murayama, Satoh, Oka, Imanishi, & Noishikl, 1988; Schmidt & Baier, 2000).

Fig. 5 presents the photomicrographs of the fresh and fixed porcine aortas, stained with Masson. We can find that, as the critical elements for the structural integrity, the microcosmic structures of collagen fibers were all preserved well after fixation and thus this structure similar to that of fresh tissues was suitable for the adhesion and proliferation of cells. Additionally, the microcosmic structure of elastic fibers was also largely preserved (Fig. 6). As a result, we can conclude that, as the same as glutaraldehyde and polyepoxy, ADA was an effective crosslinking reagent without disintegrating the natural structure of the tissues.

3.6. Cytoxicity study of ADA-fixed tissues

Fig. 7 illustrates the effect of extraction liquid of various fixed tissues in the study on proliferation of the fibroblast cells. It was noted that the extraction liquid of glutaraldehyde-fixed and polyepoxy-fixed tissues exhibited obvious cytotoxicity compared to the negative control. On the other hand, the extraction liquid of ADA-fixed tissues was significantly superior to all other counterparts. The result indicated that both glutaraldehyde and polyepoxy were cytotoxic and the cytotoxic effect of the polyepoxy was lower than that of glutaraldehyde, which was also confirmed by Nishi et al. (1995). In contrast, the extraction liquid of ADA-fixed tissues is beneficial to the growth and proliferation of fibroblasts, which means, ADA is a crosslinking reagent with low cytotoxicity.

In clinical practice, glutaraldehyde has been used extensively as a crosslinking reagent. The glutaraldehyde-fixed tissues have also performanced well in vitro properties, such as in mechanical properties. However, the high cytotoxicity of glutaraldehyde has been animadverted all along. In this study, the tissues were fixed by ADA using its aldehyde functional group in the same way as glutaraldehyde. Besides the ideal crosslinking property, the cytotoxicity of ADA was also very low. This may be due to that ADA itself is derived from the naturally occurring polysaccharide. Additionally, the molecular weight of ADA (about 10,000) is significantly larger than glutaraldehyde (100), which might make the diffusion of ADA into the cell more difficult than glutaraldehyde. Therefore, ADA is more difficult to react with proteins or polysaccharides present inside the cells, and thus result in less cell death than glutaraldehyde.

4. Conclusion

In conclusion, this work demonstrated the feasibility to use ADA as a novel crosslinking reagent in the fixation of biological tissues. The results suggest that the fixation index of ADA-fixed tissues was significantly superior to their glutaraldehyde-fixed and polyepoxy-fixed counterpart. Meanwhile, the mechanical testing indicates that ADA was in possession of the mechanical strength comparable to glutaraldehyde and superior to polyepoxy. Histological examination of the tissues after the ADA fixation process also showed intact total framework. Moreover, the data obtained in the MTT assay implied the cytotoxicity of ADA-fixed tissue was significantly lower than that of glutaraldehyde- and polyepoxy compounds-fixed tissues. The results obtained in this vitro study demonstrate that ADA is an effective agent in the fixation of biological tissue. Moreover, further in vitro and in vivo studies will pay attention to the crosslinking stability and durability of the ADA-fixed tissue as a function of storage and chronic implant.

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