Carbohydrate Polymers 115 (2015) 54–61

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Feasibility study of the naturally occurring dialdehyde carboxymethyl cellulose for biological tissue fixation

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ARTICLE INFO

Article history:
Received 27 June 2014
Received in revised form 18 August 2014
Accepted 22 August 2014
Available online 1 September 2014

Keywords:
Dialdehyde carboxymethyl cellulose
Crosslinking characteristics
Cytotoxicity
Biological tissues

ABSTRACT

The aim of this study was to evaluate the crosslinking effect of dialdehyde carboxymethyl cellulose (DCMC) on decellularized porcine aortas. Before implanted, biological tissues must be chemically modified to avoid rapid enzymatic degradation and serious immune response. To overcome limitations like high cytotoxicity and susceptibility to calcification caused by glutaraldehyde (GA), a traditional crosslinking reagent, dialdehyde carboxymethyl cellulose (DCMC) was employed to fix biological tissues. The crosslinking characteristics and cytotoxicity of aortas fixed by DCMC were all investigated. The results indicated that DCMC-fixation significantly increased the mechanical strength and the capacity of enzymatic hydrolytic resistance of tissues. The histological examination showed that the microcosmic structures of tissues were all preserved well after DCMC fixation. In addition, the data obtained from MTT assay confirmed that the cytotoxicity of DCMC-fixed tissues was significantly lower than glutaraldehyde-fixed counterparts. In a word, the present study demonstrated DCMC might be an effective crosslinking reagent for biological tissue fixation with low cytotoxicity.

1. Introduction

Biological tissues have been widely used in fabricating bioprostheses for their constructions suitable for cell attachment, migration, and proliferation (Lu et al., 2010; Hopkins, 2005). However, the fresh tissues must be fixed or chemical modified before implanted in humans to eliminate its antigenicity and to stabilize the microstructure of tissue (Schmidt & Baier, 2000). Currently, as the most typical crosslinking reagent in clinical applications, glutaraldehyde is unsatisfactory for high cytotoxicity and susceptibility to calcification, which might impair the biocompatibility of the tissues (Xu, Li, Yu, Gu, & Zhang, 2012; Zhai et al., 2006). The degradation of glutaraldehyde-derived crosslinks and the continual release of cytotoxic aldehydes appear to contribute to prolonged toxic effects (Weadock, Olson, & Silver, 1983; Huang-Lee, Cheung, & Nimni, 1990). To overcome these disadvantages, a crosslinking reagent with low cytotoxicity, stable crosslinking structure and ability to promote cell adhesion and proliferation is eagerly required.

Attempting to achieve this goal, polysaccharides are selected as superior materials for tissue fixation since they are biochemically similar to extracellular matrix (ECM) which can improve cell/substrate interactions (Shelke, James, Laurencin, & Kumbar, 2014; Colvin et al., 2011; Huisman, Schols, & Voragen, 1999). Unfortunately, there were no suitable functional groups in polysaccharides to fix tissues. So, reactive aldehyde groups are needed to be introduced in to react with primary amines within tissues for further crosslinking (Sung, Shih, & Hsu, 1996). After being selectively oxidized with periodate, multiple functional aldehyde groups formed by specifically cleaving the C2–C3 bond of the 1,4-glucan unit (Xu et al., 2012, 2013a,b; Kristiansen, Potthast, & Christensen, 2010; Mu, Guo, Li, Lin, & Li, 2012; Zhang, Wang, Zhang, Yang, & Wang, 2010). Being rich in reactive aldehyde groups, oxidized polysaccharides may react with free amino groups in biological tissues in the same way as glutaraldehyde. The aforementioned assumptions prompted us to evaluate the feasibility of using oxidized polysaccharides as a novel crosslinking reagent for the fixation of biological tissues.

Cellulose, as the most widely spread polysaccharides and an excellent source of renewable polymeric material, displays the potential value suitable for implant materials due to the excellent biocompatibility and biodegradability (Ali, El-Rehim, Kamal, & Hegazy, 2008; Jiang, Li, Zhang, & Wang, 2009; Park, Joo, & Kim, 2006; Müller et al., 2006). Until now, cellulose has received a great deal of attention from, for example, pharmaceutical, medical, food industries as well as biomaterials for implantation (Li, Wu, Mu,

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http://dx.doi.org/10.1016/j.carbpol.2014.08.051
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& Lin, 2011). In this work, carboxymethyl cellulose (CMC) was partially oxidized by sodium periodate to form dialdehyde carboxymethyl cellulose (DCMC) with a large number of multiple functional aldehyde groups. To evaluate the crosslinking characteristics and toxicity of biological tissues fixed with various concentration of DCMC, the mechanical properties, fixation index, anti-enzymatic degradation, ultrastructure and cytocompatibility of them were investigated. Glutaraldehyde (GA), the predominant chemical reagent in pretreating natural biological tissues in clinical applications, was used as control.

2. Materials and methods

2.1. Materials

Analytical grade sodium periodate ($NaIO_4$; 99.5%) and carboxymethyl cellulose sodium (viscosity of the 2% (w/v) CMC in water was ≥1200 mPa s) were obtained from Kelong Chemical Reagent Company (Chengdu, China). Triton X-100 was obtained from Amresco Co. (USA). Glutaraldehyde and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DNaseI and RNseA were obtained from Aladdin Co (Shanghai, China). Collagenase type I, DMEM, trypsin, streptomycin and penicillin were supplied by Gibcobrl (Grand Island, NY, USA). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT, USA). The other chemicals used were also of analytical grade (99.5%) and further purification was unnecessary.

2.2. Preparation of dialdehyde carboxymethyl cellulose (DCMC)

After 10 g carboxymethyl cellulose sodium was dissolved completely in 200 ml distilled water, 100 ml periodate solution (0.11 g/ml) was added under continuously magnetic mechanical stirring. Subsequently, the pH was adjusted to 3.0 by addition of sulfuric acid solution (1 M) (Kristiansen et al., 2010; Li et al., 2011). Add excess ethylene glycol to decompose the remaining periodate after the mixture was stirred in the dark at 37 °C for 4 h (Kim, Wada, & Kuga, 2004). The oxidized product, referred to DCMC, was dialyzed immediately against distilled water for three days until the dialyzate was periodate free. The solution was then lyophilized to obtain the product. The degree of oxidation of DCMC are determined through hydroxylamine hydrochloride/sodium hydroxide method and the value of oxidation degree (OD) was evaluated as following formula:

$$\mathrm{OD} = \frac{n(\mathrm{CHO})/2}{w_{\mathrm{DCMC}}/211} \quad (1)$$

where 211 is approximately the molecular weight of repeating unit in DCMC (Scheme 1).

2.3. FTIR spectroscopy

To characterize the chemical structure of DCMC, FTIR spectra of native cellulose and DCMC was recorded by a Nicolet 560. About 2.0 mg dry samples and 20 mg potassium bromide (KBr) were pressed as pellets using a hydraulic press carver. The measurements were carried out under the resolution of 4 cm^{-1} in the wave number ranging from 400 cm^{-1} to 4000 cm^{-1}.

2.4. Decellularization and crosslinking

Fresh porcine aortas used as raw materials were procured from a local slaughterhouse. To prevent degradation, the procured aortas were transported in 4 °C saline solution and the maximum time period between retrieval and tissue fixation beginning was less than 6 h (Chen, Sung, Liang, & Chang, 2002). After removing the excess blood by rinsing with fresh saline, the adherent fat on the aortas surface was carefully trimmed without damaging the tissue structure. Subsequently, decellularized tissues were obtained according to previously reported methods (Xu et al., 2012). Briefly, the aortas were incubated in PBS containing 0.1% trypsin and 0.02% EDTA at 37 °C for 4 h with continuous shaking. Then, the porcine aortas were immersed in a 1% solution of Triton X-100 for 48 h for cell lysis. Finally, RNaseA (0.02 mg/ml) and DNaseI (0.2 mg/ml) were introduced to treat these tissues at 37 °C for 4 h with constant stirring to remove cellular components.

Washed with sterile PBS, the decellularized aortas were fixed by varieties concentration of DCMC solution (0.25, 0.5, 0.75, 1, 2.5 mg/ml) in dimethyl sulfoxide (DMSO) at 37 °C for 3 days under constant shaking. Meanwhile, the decellularized aortas crosslinked by 0.625% glutaraldehyde (GA) solution were served as control.

To reduce reverse reaction, the cross-linking bonds could be stabilized by a reduction reaction with sodium borohydride (Han, Lee, & Kim, 2010). After Schiff-base had formed between DCMC and tissues during tissue-fixation, the samples were immersed in 0–4.0 wt% sodium borohydride solutions at 4 °C for 3 h with continuous agitating, then washed with saline for several times to remove residual inorganic salt and followed by crosslinking characteristics tests.

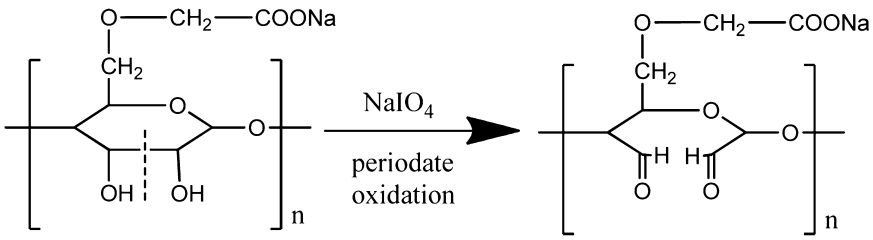
2.5. Fixation index determination

The degree of crosslinking, determined by ninhydrin (NHN) assay, reveals the percentage of amino groups reacting with aldehyde groups on the dialdehyde cellulose consumed. As is reported, after specimens being heated in NHN solution, the amount of free amino groups in tissue is proportional to the optical absorbance of the solution (Yu, Wan, & Chen, 2008). Briefly, samples of each group ($n = 6$) were taken out at predetermined fixation periods (from 15 min to 72 h). The tested tissues were lyophilized for 24 h and then weighed. After heating in NHN solution to boiling for 20 min, lyophilized tissues were taken out and optical absorbance of the solution was measured by spectrophotometer (UV-752, Shanghai) at 570 nm. Glycine at various known concentrations was used as a standard. The fixation index was evaluated by following formula:

$$\mathrm{FI}(\%) = \frac{(\mathrm{NHN\ \ reactive\ \ amine})_{\mathrm{fresh}} - (\mathrm{NHN\ \ reactive\ \ amine})_{\mathrm{fixed}}}{(\mathrm{NHN\ \ reactive\ \ amine})_{\mathrm{fresh}}} \quad (2)$$

2.6. Biomechanical test

The mechanical determination was performed according to our previously described methods (Xu et al., 2013a,b). In preparation of specimens, aortas were longitudinally opened and cut along the preferential orientation of collagen fibers to yield 40 mm × 4 mm rectangular slab. The accurate width and thickness of each sample was measured using a micrometer. Tensile strength of tissue strips obtained from each group ($n = 5$) were tested by Instron material testing machine (Instron Co., USA) at a constant speed of 10 mm/min. As soon as fracture occurred, the first decrease in load of each sample was detected and the ultimate tensile strain together with the ultimate tensile stress were recorded before failure. After measurement, the ultimate elastic modulus was determined from the stress–strain curves.



Scheme 1. Preparation process of DCMC.

2.7. In vitro enzymatic degradation

To evaluate the capacity of enzymatic hydrolytic resistance of fixed tissues, vitro enzymatic degradation was carried out according to a reported method by Sung, Chang, Liang, Chang, and Chen, (2000), Yao et al. (2004). Collagen fibers were the most important component of fundamental structural framework of biological tissues. Therefore, collagenase I, with an activity of 125 U/mg solid, was used to treat test samples. Briefly, DCMC-fixed, GA-fixed aortas along with fresh ones were immersed in the 250 U/ml collagenase/PBS solution, incubated at 37 °C for selected duration (0.5 h, 1 h, 3 h, 6 h, 12 h and 24 h) under continuous shaking. Enzymatic degradation was terminated at desired duration by adding 10 mM EDTA solution. Before and after enzymatic degradation, all the samples were lyophilized and weighted to calculate the weight loss percentage (W%) according to the following formula:

$$\Delta W\% = \frac{W_0 - W_t}{W_0} \times 100\% \quad (3)$$

where W_0 represents the original weight of each sample and W_t represents the weight of the corresponding sample after degradation with collagenase I.

2.8. Light microscopy

To observe the total framework of crosslinked biological tissues, decellularized and crosslinked tissues were fixed in 4% formaldehyde for more than three days (Xu et al., 2012). Subsequently, the specimens were examined histologically by Masson staining for collagen fibers and Verhoeff iron hematoxylin staining for elastic fibers. The stained sections were observed by light microscopy (Olympus Corporation, Japan).

2.9. Cytocompatibility study of fixed tissues

The effect of DCMC-fixed tissues on cell proliferation was evaluated in vitro using a mouse-derived established cell line of L929 fibroblasts (Xu et al., 2013a,b). Extraction liquid was obtained after the sterilized samples being immersed and incubated in saline at 37 °C for 24 h in 5% CO_2. Subsequently, L929 fibroblasts were seeded in 96-well plates at the density of 2×10^3 cells/well in 100 μl Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Meanwhile, 100 μl extraction liquid obtained from various fixed tissues was added into each well. The cell culture was maintained in a humidified incubator at 37 °C with 5% CO_2 in air and each sample was photographed using an inverted light microscope on the 4th day to observe the morphology of L929. Furthermore, surviving cell number was indirectly analyzed using 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 1, 4 and 7 days, respectively. L929 from passage 3 was used for the cell proliferation experiment.

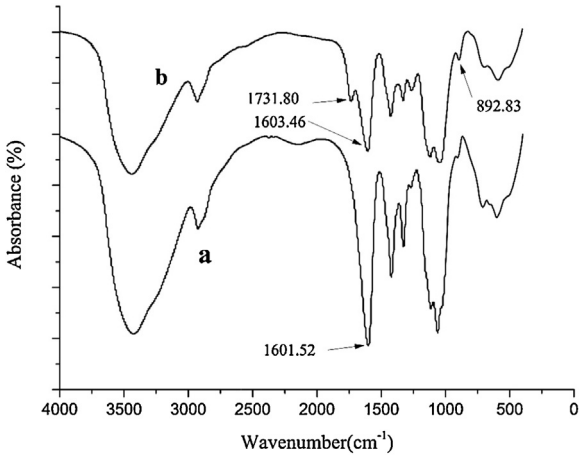


Fig. 1. FT-IR spectra of a CMC (a) and DCMC (b). The major absorbance for the functional groups are noted.

3. Results and discussion

3.1. Preparation and assessment of DCMC

Fig. 1 shows the FTIR spectra of CMC and DCMC. The cleavage of C2–C3 bond results in the formation of two multiple functional aldehyde groups per glucose unit. The appearance of absorption band at 1731.80 cm^{-1}, the most characteristic band of C=O vibrations in aldehyde groups, indicates the aldehyde groups of DCMC. In the fingerprint region, a new infrared band at 892.83 cm^{-1} was associated with the formation of hemiacetals between aldehyde groups and the hydroxyl groups of unoxidized residues (Mu et al., 2012; Fan, Lewis, & Tapley, 2001). Consequently, aldehyde group has been introduced successfully through periodate oxidation of CMC. The measured degree of oxidation was 66.8%, which was selected as the optimal oxidation degree for perfect crosslinking affect.

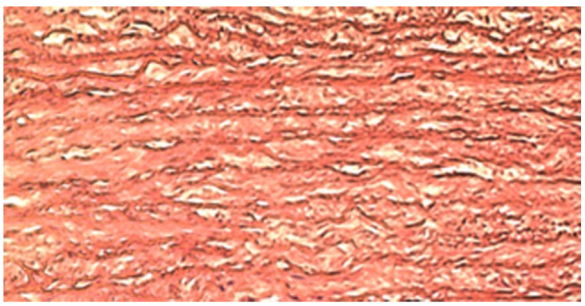


Fig. 2. The decellularized porcine aortas.

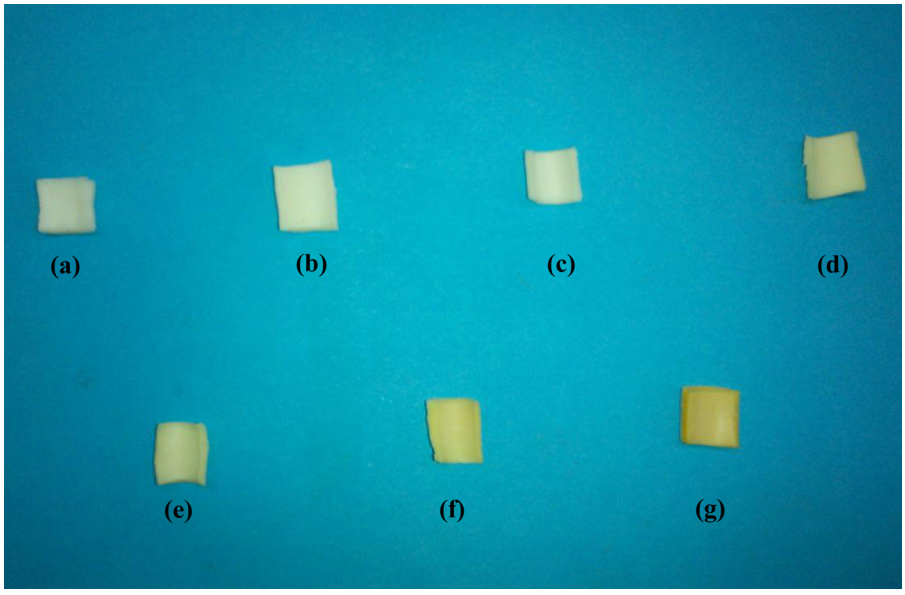


Fig. 3. Digital image of non-crosslinked and crosslinked porcine aortas. Sample (a) is non-crosslinked tissue; samples ((b)–(f)) are crosslinked by DCMC solution with a concentration of 0.25, 0.5, 0.75, 1, 2.5 mg/ml; sample (g) is crosslinked by 0.625%GA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

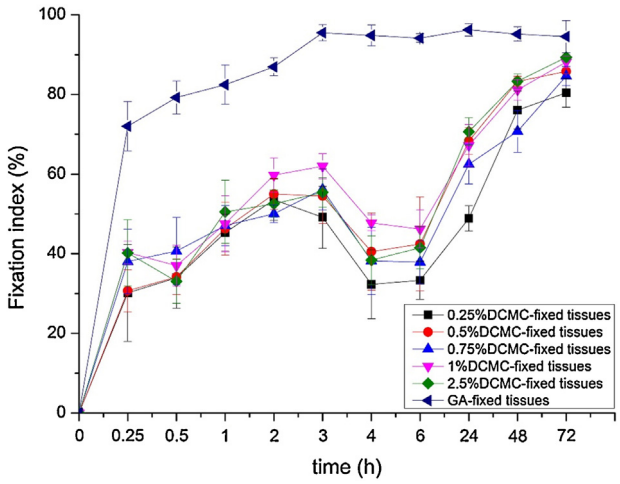


Fig. 4. Fixation index of the tissues fixed with GA and various concentration of DCMC.

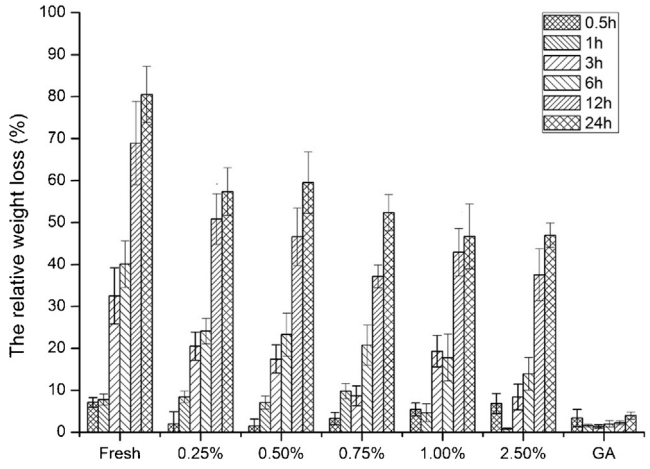


Fig. 5. The relative weight loss of fresh, glutaraldehyde-fixed and 0.25%, 0.5%, 1%, 2.5% DCMC-fixed tissues during enzymatic degradation in vitro at different time points.

3.2. Decellularization and fixation

As shown in Fig. 2, after decellularization procedure, there were no residual cellular components in the tissues which might cause the immune response in vivo implant. Therefore, the antigenicity of tissues can be reduced. Moreover, it can form excellent microporous structure suitable for cell attachment, proliferation, and differentiation. Fresh, glutaraldehyde-fixed and DCMC-fixed porcine aortas were shown in Fig. 3. With DCMC concentration increasing, the color of porcine aortas changed gradually from milky white to yellow, while the GA crosslinked sample became light yellow. Compared with fresh aortas, DCMC-fixed tissues were as flexible and soft as natural tissues, but the GA-fixed one was a little rigid and dehydrant. It can be confirmed that DCMC was superior to GA in maintaining aortas natural configuration.

Table 1
Mechanical properties of porcine vascular tissues ($X \pm S$, $n = 5$).

Treated samples	Ultimate tensile strain (%)	Ultimate tensile stress (MPa)	*E*-modulus (MPa)
Fresh	139.74 ± 11.64[#]	1.11 ± 0.23[#]	1.51 ± 0.70[#]
0.25%DCMC-fixed	101.73 ± 13.93[*]	1.03 ± 0.19	2.17 ± 0.617
0.5%DCMC-fixed	74.41 ± 14.03[*#]	1.24 ± 0.12	2.42 ± 0.65
0.75%DCMC-fixed	74.40 ± 14.02[*]	1.35 ± 0.18	2.43 ± 0.65
1%DCMC-fixed	87.48 ± 15.54[*]	1.43 ± 0.07	2.32 ± 0.27
2.5%DCMC-fixed	73.71. ± 19.87[*#]	1.55 ± 0.48	3.06 ± 1.06[*]
GA-fixed	89.93 ± 19.78[*]	1.91 ± 0.45[*]	2.68 ± 0.86[*]

[*] $P < 0.05$ compared with fresh tissues.
[#] $P < 0.05$ compared with GA.

3.3. Fixation index determination

The fixation index is usually used to evaluate the percentage of amino groups reacted with aldehyde groups forming stable

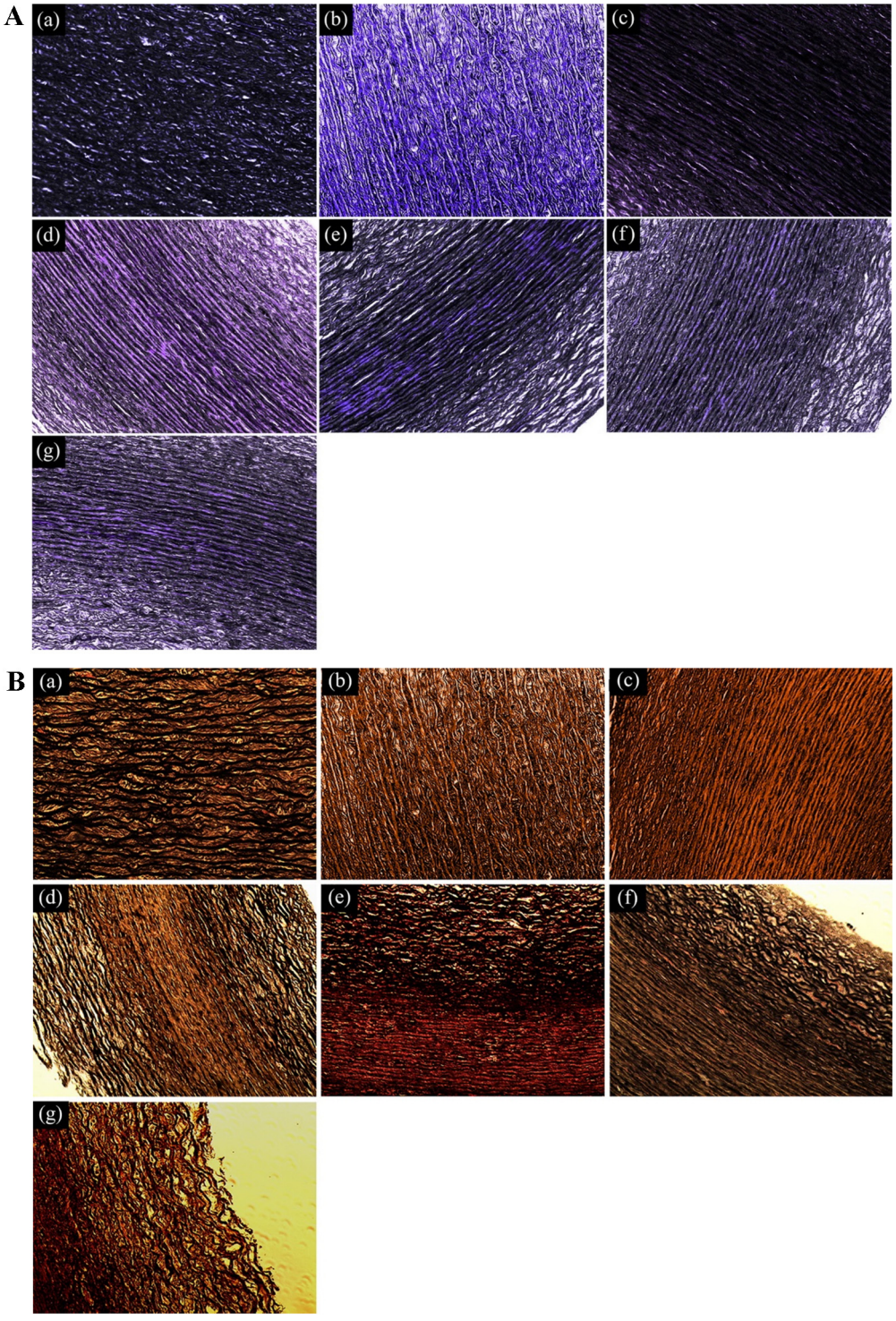


Fig. 6. (A) Photomicrographs (Masson staining, 200 × magnification) of the (a) fresh specimen (b) GA-fixed specimen and ((c)–(g)) 0.25%, 0.5%, 0.75%, 1%, 2.5% DCMC-fixed specimens, respectively. (B) Photomicrographs (Verhoeff iron hematoxylin staining, 200 × magnification) of the (a)fresh specimen (b)GA-fixed specimen and ((c)–(g)) 0.25%, 0.5%, 0.75%, 1%, 2.5% DCMC-fixed specimens, respectively.

cross-linking bridge structure. A lower level of free amino groups left often implies a higher fixation index in fixed tissues (Sung et al., 1996). As is shown in Fig. 4, the fixation index of the GA-fixed tissues increased more rapidly than DCMC-fixed ones, which illustrated that the initial fixation rate of GA was faster than that of DCMC. Moreover, the fixation index of GA-fixed tissues nearly reached maximum (>90%) after 2 h fixation. On the contrary, the maximum (88%) of fixation indices belonging to the tissues fixed by various concentration of DCMC was achieved at the end of fixation (72 h). As indicated in the figure, though a longer fixation time was required for DCMC to make the crosslinking degree similar to GA, the ultimately fixation effect was comparable. Among the tissues fixed with DCMC, the fixation index increased with fixation time proceeding, and that of tissues crosslinked with DCMC in concentration of 1% and 2.5% were higher than other counterparts at any time points and finally reached 88.15% at the 3rd day, which

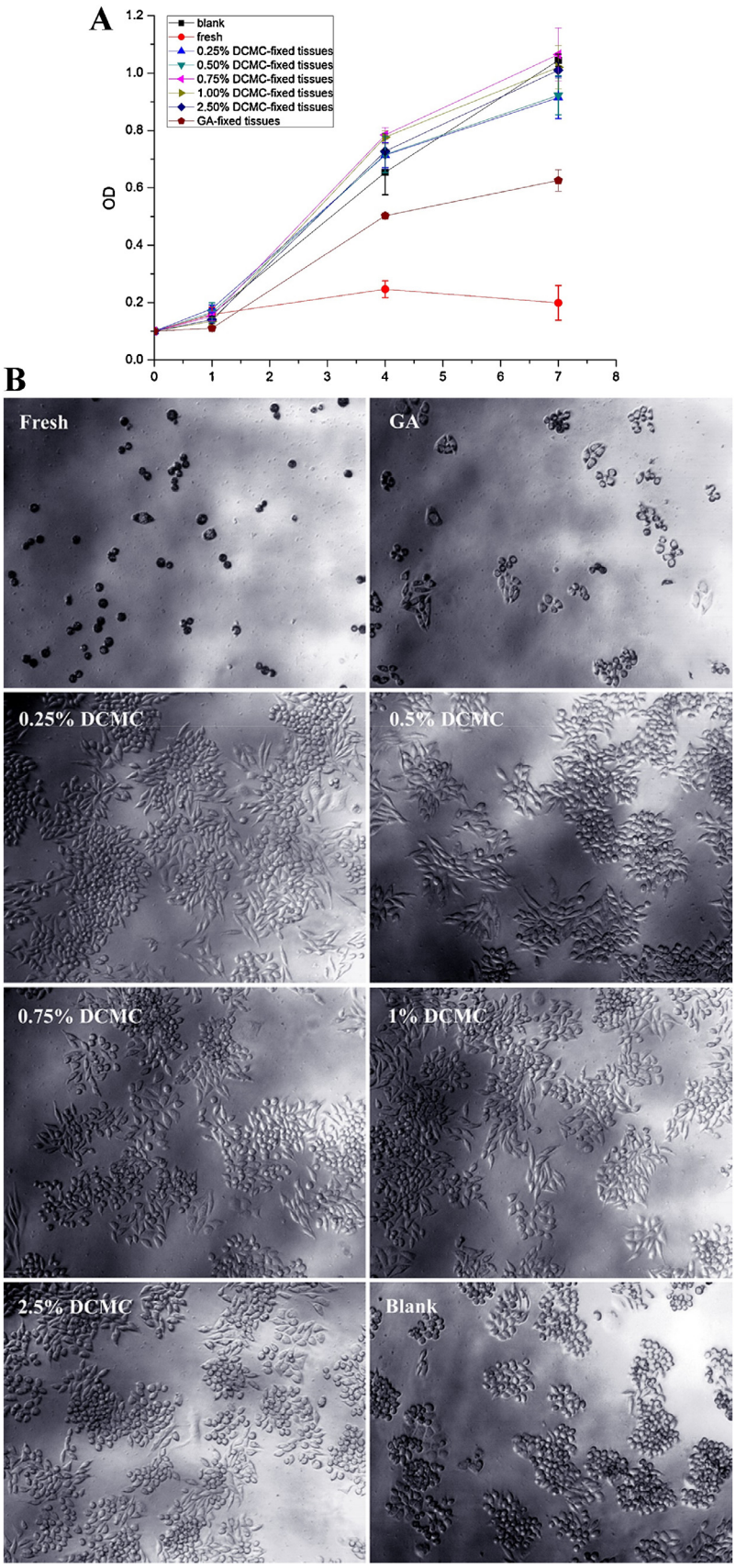


Fig. 7. (A) Proliferation of the L929 fibroblasts cultured in the extraction liquid of various fixed tissues. (B) Observation of L929 cell morphology cultured in the extraction liquid of various fixed tissues: (a) fresh specimen (b) GA-fixed specimen ((c)–(g)) 0.25%, 0.5%, 0.75%, 1%, 2.5% DCMC-fixed specimens and (h) blank control, respectively.

was close to that of GA-crosslinked (0.625%) samples. Therefore, the two concentrations were selected as the optimal concentration for DCMC fixation.

3.4. Biomechanical properties of crosslinked aortas

In order to serve as prosthesis with function during implantation, adequate mechanical strength is required for fixed tissue (Zhai et al., 2006; Sung, Liang, Chen, Huang, & Liang, 2001). As shown in Table 1, DCMC-fixation significantly increased the mechanical strength of decellularized porcine aortas. The values of "Ultimate tensile stress" and "*E*-modulus" for GA-fixed and DCMC-fixed tissues were significantly higher than that of the fresh ones, while the values of "Ultimate tensile strain" decreased, which suggested fresh tissues a better elasticity. Based on values above, we can obviously conclude the mechanical strength of tissues increased when treated with crosslinking reagents, and that of DCMC- and GA-fixed tissues were comparable.

The ultimate tensile stress of DCMC-fixed tissues increased with the increasing DCMC concentration for fixation and achieved maximum of 1.55 ± 0.48 MPa at 2.5% DCMC, which was exactly in accordance with the result of fixation index. The *E*-modulus of DCMC-fixed tissues was also concentration-dependant increasing in response to the increasing DCMC concentration and the highest level was achieved at 2.5% DCMC. The increase of tensile stress of DCMC-fixed tissues also confirmed that effective crosslinking formed from reaction between the functional dialdehyde groups in DCMC and the amino groups in tissues.

3.5. In vitro enzymatic degradation

Fig. 5 presents the relative weight loss of non-, GA- and DCMC-crosslinked tissues during enzymatic degradation process in vitro at different time points. As illustrated in the figure, the relative weight loss of all crosslinked tissues was significantly lower than that of fresh aortas at any time points. With hydrolysis proceeding, the weight of the residual specimens decreased continuously. However, the unfixed-tissues were hydrolyzed more rapidly and extensively than fixed ones. After the first 30 min's digestion, the fresh aortas were hydrolyzed by 7.82%, which was similar to the fixed samples. But when digested for 24 h, the fresh aortas were hydrolyzed by 80.52%, while 0.625% GA-fixed tissues or 0.25%, 0.5%, 1% and 2.5% DCMC-crosslinked tissues were hydrolyzed by only 3.98%, 57.35%, 59.50%, 52.32%, 46.66% and 46.96%, respectively. As shown in Fig. 5, the capacity of enzymatic hydrolytic resistance was concentration-dependant increasing in response to the increasing DCMC concentration and reached maximum at 2.5%, which was selected as the optimal concentration for fixation. The resistance against enzymatic degradation of DCMC-fixed tissues, lies between fresh and GA-fixed samples, were good in the early stage while later degraded properly with elapsing hydrolytic time to generate some cavities that was beneficial to ingrowth of new-formed tissues (Xu et al., 2013a,b). This enabled the DCMC-fixed tissues suitable for tissue engineering scaffolds. The remarkable resistance to enzymatic hydrolytic degradation shown in the GA- and DCMC fixed tissues probably resulted from the cleavage sites of collagen being hidden or modified by the reaction of crosslinking between the free amino groups and aldehyde groups (Lu et al., 2010; Zhai, Lu, Chang, Zhou, & Zhang, 2010). Moreover, a compact structure in fixed tissues was formed, which was an obstacle for enzymatic penetration into fixed-tissues (Gu et al., 2011).

3.6. Ultrastructure of DCMC-fixed tissues

To observe the total framework of the crosslinked vascular tissues, histological examination was performed after fixation process. Fig. 6(A) and (B) presents the photomicrograph of porcine aortas stained with Masson and Verhoeff iron hematoxylin. The microscopic structure of collagen fibers and elastic fibers, primary components of natural extracellular matrices, were all preserved well after DCMC-fixation. The ultrastructure of tissues treated with various concentration of DCMC was similar to that of fresh tissues, which resulted in its properties suitable for the adhesion and proliferation of cells (Xu et al., 2012; Gu et al., 2011).

All data mentioned above forcefully demonstrated that, in the similitude of glutaraldehyde, DCMC was an effective crosslinking reagent without damaging the natural structure of the tissues.

3.7. Cytocompatibility of the fixed tissues

After 1, 4 and 7 days of culture, the cell proliferation in the extraction liquid of tissues fixed with various concentration of DCMC was determined by MTT assay. Fig. 7(A) illustrates the optical density readings of the L929 fibroblasts cultured in the extraction liquid of fixed tissues and Fig. 7(B) presents the morphology of L929 fibroblasts cultured in extraction liquid on the 4th day. Compared to the negative control, GA-fixed and decellularized tissues exhibited an obvious inhibition effect on cell proliferation: the optical density values decreased dramatically on the first day and the morphology of cells were rounded and sparse, which were the characteristics of inactive cells. In contrast, the extraction liquid from DCMC-fixed tissues stimulated the growth and proliferation of fibroblasts significantly. Meanwhile, noticeable difference in morphology of cells was barely seen between the negative controls and tissues fixed by DCMC in various concentration, which indicated that DCMC is an effective crosslinking reagent with low cytotoxicity.

As for reaction mechanism, both GA and DCMC can react with the free amino groups of lysine, hydroxylysine or arginine residues within biological tissues through their aldehyde groups to form imino structure units (Xu et al., 2013a,b). The cytotoxicity of GA-fixed tissues is mainly attributed to the diffusion of the unreacted GA absorbed in tissue interstices (Schmidt & Baier, 2000). In contrast with GA, DCMC exhibits low cytotoxicity. We inferred that this result occurred because of the following aspects: First, DCMC was derived from naturally occurring polysaccharide, which shows low cytotoxicity. Second, the residual aldehyde groups in DCMC reacted with adjacent hydroxyl groups to form hemiacetals, which significantly reduced the cytotoxicity. Third, the molecular weight of DCMC is large enough to hinder itself diffusing into cells to react with component inside the cells (Shelke et al., 2014; Hexig, Nakaoka, & Tsuchiya, 2008).

4. Conclusion

In summary, this work demonstrated that DCMC-crosslinking was an effective method to evidently improve the mechanical properties of decellularized aortas and stabilize aorta materials to resist enzymatic degradation. This suggested that DCMC could be used for fixing tissues for the ability of forming stable crosslinks. Meanwhile, the DCMC-fixed tissues were characterized to be low cytotoxicity and low antigenicity. The data obtained from this assay implied that DCMC-crosslinked tissues were significantly less cytotoxic than GA-fixed ones. Moreover, the histological examination conformed that the microcosmic structure of tissues preserved intact after DCMC fixation. Therefore, DCMC could be an effective crosslinking reagent for fixing biological tissues thanks to its excellent crosslinking characteristics, resistance against enzymatic degradation and cytocompatibility.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30870616), the Scientific and technological project of Sichuan Province (2012SZ0015) and the Scientific Research Project of Department of Public Health of Sichuan Province (no. 100194). We also would like to thank the Analysis and Testing Center, Sichuan University (China) for their assistance of biomechanical test.

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