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In vitro enzymatic degradation of a biological tissue fixed by alginate dialdehyde

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ABSTRACT

Biological tissues must be chemically fixed before they can be implanted in humans as tissue engineering scaffolds. To provide an ideal tissue engineering scaffold material, which is biodegradable and cytocompatible, a novel crosslinking agent, alginate dialdehyde (ADA), was employed to fix biological tissues by our group. The study mainly investigated the enzymatic degradation of ADA fixed biological tissues in vitro. Glutaraldehyde, the most commonly used crosslinking agent for biological tissue fixation, was employed as a control. The results suggested that, the ADA fixation could enhance the resistance against enzymatic degradation of biological tissues effectively. Meanwhile, compared to glutaraldehyde-fixed tissues, the ADA-fixed tissues could also degrade gradually over time. Moreover, the ADA crosslinking reagent itself had a stimulatory effect on cell proliferation when at an appropriate concentration. The results obtained in this study demonstrate that ADA fixation might provide a successful example of the biodegradable scaffold materials in tissue engineering.

1. Introduction

Tissue loss or end-stage organ failure resulting from an injury or a disease is a major concern in healthcare as they are costly and devastating problems (Langer & Vacanti, 1993). Tissue engineering has now emerged as a potential alternative to tissue or organ transplantation (Chapekar, 2000). Tissue engineering is an interdisciplinary field that involves the knowledge and technology of cells, engineering scaffold materials, and suitable biochemical factors to create artificial organs and tissues, or to regenerate damaged tissues (Langer & Vacanti, 1993). Recently, the development of scaffold materials responsible for cells growing has been a major concern in tissue engineering. A major objective of the development of scaffold materials is to mimic the structure and function of extracellular matrix in a living system (Ishii, Ying, Yamaoka, & Iwata, 2004). Therefore, compared to the synthetic materials, naturally derived biological tissues show great promise in tissue engineering applications (Schmidt & Baier, 2000). Naturally derived biological tissues are composed primarily of extracellular matrix components, which can provide a natural substrate for cells attachment, proliferation, and differentiation in its native state (Chen, Harding, Ali, Lyon, & Boccaccini, 2008). Furthermore, these natural materials may also offer improved mechanical and shape compatibility compared to synthetic scaffolds. For the above mentioned reasons, when repopulated with autologous or genetically engineered cells, naturally derived biological tissues can serve as the ideal scaffold materials for tissue engineering. However, due to the immediate degradation and presence of antigenicity after implantation, these naturally derived tissues must be chemically fixed before they can be used in the clinical applications (Schmidt & Baier, 2000).

In recent years, various synthetic crosslinking reagents, such as formaldehyde, glutaraldehyde (Cheung, Perelman, Ko, & Nimni, 1985) and polyepoxy compound (Sung, Shih, & Hsu, 1996) have been widely used in the fixation of natural biological tissues, however, these synthetic crosslinking reagents are all highly (or relatively highly) cytotoxic (Nishi, Nakajima, & Ikada, 1995; Yu, Wan, & Chen, 2008). In an attempt to overcome the aforementioned cytotoxic effect of synthetic crosslinking reagents, a naturally occurring crosslinking agent, alginate dialdehyde (ADA), was developed to fix biological tissues by our group.

Alginate (ALG), an important naturally occurring carbohydrate polymer extracted from brown algae, has been widely used in a number of biomedical applications, mainly due to its high biocompatibility (Goh, Heng, & Chan, 2011; Knill et al., 2004; Isiklan, Inal, Kursun, & Ercan, 2010; Wang, Fu, Zhang, Yu, Li, & Wan, 2010). In recent years, It has been reported that alginate can be oxidized

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with periodate and form multiple functional aldehyde groups (alginate dialdehyde, ADA) (Bouhadir et al., 2001). Therefore, ADA may react with the free amino groups using its multiple functional aldehyde groups in the same way as glutaraldehyde (Xu, Li, Yu, Gu, & Zhang, 2012). On the other hand, ADA is also highly susceptible to biodegradation, therefore, has potential to be used in a variety of biomedical applications wherein biocompatibility and biodegradability are important criteria. (Balakrishnan, Lesieur, Labarre, & Jayakrishnan, 2005). These advantages of ADA in biomedical applications prompted us to use this naturally occurring crosslinking agent to fix biological tissues. In our previous study, it was found that ADA not only could crosslink the biological tissues effectively, but also had an excellent cytocompatibility for biological tissue fixation (Xu et al., 2013, 2012).

Except for the biocompatibility, degradability is generally a desired characteristic for tissue engineering scaffold materials because the second surgery to remove them (such as a heart patch) would be averted if the substrate could be removed by the physiological system of the host body (Chen et al., 2008). Theoretically, even all scaffolds used in tissue engineering are intended to degrade slowly after implantation in the patient and be replaced gradually by new tissue (Griffith & Naughton, 2002). Complete degradation of the tissue engineering scaffolds could alleviate many concerns about the long-term implant biocompatibility (Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009).

As previously mentioned, ADA was a promising crosslinking agent for biological tissue fixation, due to its excellent cytocompatibility. The present study was undertaken to further investigate the enzymatic degradation of a biological tissue fixed by ADA. In the study, fresh porcine aortas procured from a slaughterhouse were used as raw materials. Glutaraldehyde, the most commonly used crosslinking agent for biological tissue fixation (Jayakrishnan & Jameela, 1996), was employed as a control.

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 and Diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). EDTA and Triton X-100 were obtained from Amresco Co. (USA). DNaseI and RNseA were obtained from Aladdin Co (Shanghai, China). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, USA). Collagenase type I, DMEM, trypsin, penicillin and streptomycin were purchased from Gibcobrl (Grand Island, NY, USA). Sodium periodate and all other chemicals of the analytical reagent were purchased from Kelong Co. (Chengdu, China).

2.2. Preparation of ADA

ADA was prepared according to our previously reported method (Vieira, Cestari, Airoldi, & Loh, 2008; Wang et al., 2010a,b). The sodium alginate (5 g) was dissolved in 200 ml distilled water and 50 ml pure ethanol by prolonged magnetic stirring in a beaker. And then 5.7 g of sodium periodate were added to the solution in the dark at room temperature to prepare the product. After 24 h, the reaction was neutralized by the addition of 10 ml ethylene glycol to reduce the excess periodate. The reaction mixture was continuously stirred under dark for 2 h. 5 g of sodium chloride was then added to the solution, followed by precipitation with 800 ml of ethanol. The precipitates were then dissolved in about 100 ml distilled water again and reprecipitated by the addition of 600 ml ethanol. This process was repeated three times. Furthermore, the product solution was dialyzed using dialysis tube (MWCO, 3500) against distilled water with several changes of water until it was free from periodate (Balakrishnan et al., 2005). The dialyzate was then lyophilized to obtain the product.

2.3. Decellularization and crosslinking process

Decellularized porcine aortas tissues were obtained according to previously described methods (Yu, Liu, Xu, & Wan, 2010). Briefly, fresh porcine aortas were procured from a local slaughterhouse and treated with 0.1% trypsin and 0.02% EDTA in PBS solution for 4 h at 37 °C. Then, the aortas tissues were washed with sterile PBS, followed by treatment in a hypotonic tris solution with 1% Triton X-100 for 48 h. Finally, the tissues were further incubated with RNaseA (0.02 mg/ml) and DNaseI (0.2 mg/ml) for 4 h at 37 °C.

After washing with sterile PBS solution, the decellularized aortas were fixed in a 15% ADA solution, which was buffered with phosphate-buffered saline (pH 7.4). Meanwhile, the samples fixed with 0.625% glutaraldehyde solution (buffered with phosphate-buffered saline, pH 7.4) were used as controls. The samples of each group were all fixed at 37 °C for 72 h under continuous shaking (Xu et al., 2013).

2.4. In vitro enzymatic degradation

To assess the resistant of all the samples to enzymatic degradation, in vitro enzymatic degradation of porcine aortas tissues was evaluated according to a modified method used by Sung, Chang, Liang, Chang, and Chen (2000) and Yao et al. (2004). The degradation was performed using collagenase type I digestion (with an activity of 125 U/mg solid). Fresh tissues, GA-fixed tissues and ADA-fixed tissues were immersed in the 250 U/ml collagenase/PBS solution, incubated at 37 °C under continuous shaking. The degradation was discontinued at predetermined intervals (30 min, 1 h, 3 h, 6 h, 12 h and 24 h) by an addition of 10 mM EDTA solution. In addition, all the samples before and after degradation were photographed using a digital camera.

2.5. The weight loss during degradation process

In the weight loss study, all the tested samples were first lyophilized and then weighed before and after enzymatic degradation. The degradation rate or weight loss percentage ($\Delta W\%$) was then calculated according to the formula:

$$\Delta W\% = \frac{W_0 - W_t}{W_0} \times 100\%,$$

where W_0 represents the initial weight of each sample and W_t represents the weight of the corresponding sample after enzymatic degradation.

2.6. Mechanical testing

The mechanical properties of samples before and after degradation were all examined based on our previous described methods (Yu et al., 2010). Briefly, to prepare specimens for tensile strength testing, each sample from individual group was trimmed as a test strap of 4 mm × 40 mm along collagen fiber, then the thickness and width of the sample was obtained using a micrometer. Five tissue straps in each group were extended on an Instron material testing machine (Instron Co., USA) from 0 g load until the tissue strip ruptured at a constant speed of 10 mm/min. After measurement, the ultimate tensile stress and the ultimate tensile strain were recorded before failure. During testing, the tissue strips were kept in air.

2.7. Light microscopy

All the samples used for light microscopy were fixed in 4% formaldehyde for at least 3 days and prepared for histological examination. In the examination, the samples were embedded in paraffin, sectioned into thicknesses of 5 mm, and then stained with hematoxylin and eosin (H&E). Meanwhile, to specially observe the structure integrity of the collagen fibers in tissues, the samples were further examined histologically by Masson staining. The stained sections of each test sample were examined using light microscopy. (Olympus Corporation, Japan).

2.8. Scanning electron microscopy (SEM)

To observe enzymatic degradation effect more clearly, all the samples were performed SEM observation. The samples first were fixed with 3% glutaraldehyde in PBS solution. Subsequently, the samples were dehydrated in a graded series of ethanol solutions, critical-point dried with carbon dioxide, and spattered with gold film. The examination was performed with a scanning electron microscope (JSM-7500F, JEOL).

2.9. Effect of crosslinking reagents on the cells proliferation

The cytotoxicity of crosslinking reagents was evaluated in vitro using a mouse-derived established cell line of L929 fibroblasts. The cells were trypsinized and inoculated into 96-well plates at the density of 2×10^3 cells/well in 100 μl DMEM medium. After 1 day, the medium in wells was respectively replaced with medium supplemented with glutaraldehyde in a serial concentration of 0.0156, 0.156, 0.315, 0.625, 1.25 mg/ml (Zhai et al., 2006) or ADA in a serial concentration of 125, 250, 375, 500, 1000 mg/ml ($n = 6$). The cell culture was performed at 37 °C in humidified atmosphere (5% CO_2 in 95% air). Using the MTT assay, the viable cells cultured in each well were determined at 2, 4, and 6 days after cell seeding. Details of the methodology used in the MTT assay were previously described (Yu et al., 2008). Briefly, at the end of incubation period, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline) was added to each well and then the plate was incubated at 37 °C to allow the formation of insoluble formazan crystals. After 4 h, the liquid was aspirated and 200 μl of DMSO was added to each well, followed by constant shaking at room temperature to dissolve the dark blue crystals, the product of deoxidized MTT yielded by mitochondrial dehydrogenases of living cells. After 10 minutes, the optical density (OD) at 492 nm was measured using a Microplate Reader (Model550, Bio Rad Corp.).

3. Result and discussion

3.1. In vitro enzymatic degradation

Naturally derived biological tissues used in our study are composed primarily of extracellular matrix components, which can provide a natural substrate for cell attachment, proliferation, and differentiation in its native state. Alternatively, these natural extracellular matrices of soft tissues are mainly composed of various collagens. So that was the reason why we used Collagenase type I for our in vitro enzymatic degradation study.

Fig. 1 gives a photograph of representative fresh, glutaraldehyde-fixed, ADA-fixed tissues before and after enzymatic degradation respectively. It was observed that the fresh tissues became significantly thinner and more shrinkable after enzymatic degradation [Fig. 1(a,a‘)], while the glutaraldehyde-fixed and ADA-fixed tissues almost remained integral throughout the entire enzymatic degradation process [Fig. 1(b,b‘,c,c‘)].

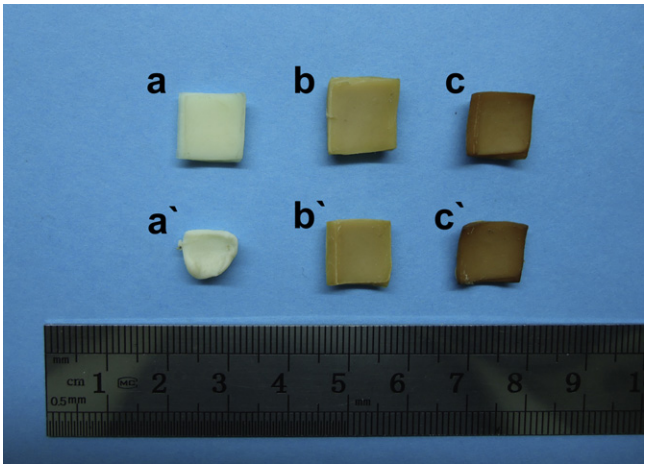

Fig. 1. Photographs of the representative (a) fresh tissues, (b) glutaraldehyde-fixed tissues, (c) ADA-fixed tissues before degradation; (a‘) fresh tissues, (b‘) glutaraldehyde-fixed tissues, (c‘) ADA-fixed tissues after degradation.

Naturally derived biological tissues could be a favorite substrate for tissue engineering, because they can provide better constructions for adhesion and growth of cells than synthetic materials. Nonetheless, previous studies have shown these biological tissues are readily degraded by various proteolytic enzymes after implantation in clinic. Therefore, biological tissues must be chemically fixed before they can be implanted in humans (Schmidt & Baier, 2000). The observed results revealed that the glutaraldehyde-fixed and ADA-fixed tissues could resist the enzymatic degradation compared to the fresh tissues, which suggested that effective ADA fixation process might protect the tissues from enzymatic degradation in vitro.

3.2. The weight loss during degradation process

Fig. 2 illustrates the relative weight loss of fresh, glutaraldehyde-fixed and ADA-fixed tissues during enzymatic degradation process in vitro at different time points. It was obvious that the relative weight loss of all the test samples increased while the hydrolytic time proceeded. Alternatively, the fresh tissues degraded more rapidly and extensively compared to the fixed tissues. It was noted that the fresh tissues were hydrolyzed by 12.08% of the original weight after 30 min digestion and was hydrolyzed by 79.38% after 24 h digestion, while the glutaraldehyde-fixed tissues were hydrolyzed only 4% while the ADA-fixed tissues were

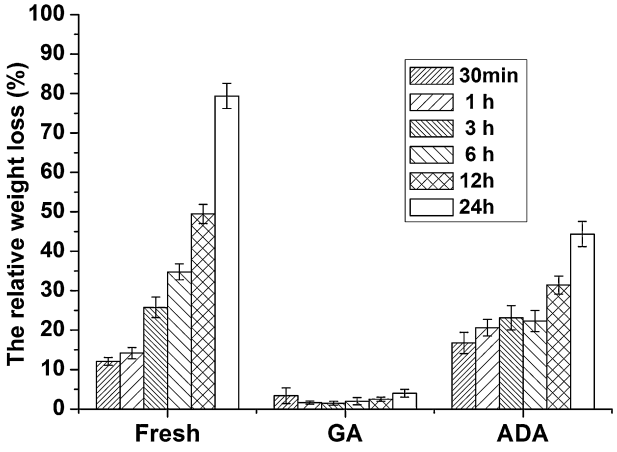

Fig. 2. The relative weight loss of fresh, glutaraldehyde-fixed and ADA-fixed tissues during enzymatic degradation in vitro at different time points.

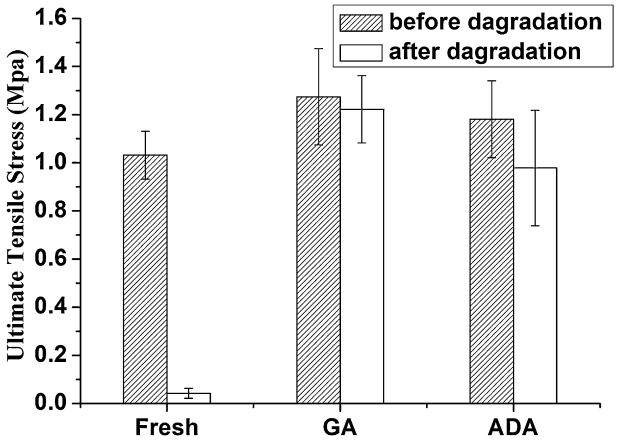

Fig. 3. The ultimate tensile stress of the test samples obtained before and after enzymatic degradation.

hydrolyzed 44.38% even after 24 h digestion. This relative weight loss of test tissues with increasing duration of degradation reflected the respective degradation rate and the resistance against enzymatic degradation of the test samples. Hence, the results obtained in this test suggested that the degradation rate and the resistance against enzymatic degradation of ADA-fixed tissues were between the fresh tissues and glutaraldehyde-fixed tissues.

3.3. Mechanical testing

Fig. 3 presents the ultimate tensile stress of all test samples obtained before and after enzymatic degradation. As illustrated in the figure, the ultimate tensile stress for the fixed tissues was significantly higher than that of the fresh tissues before degradation. Previous studies have shown that glutaraldehyde and ADA could use their aldehyde functional groups to crosslink collagen fibers in the same way. The network crosslinking structure was created intramolecularly and intermolecularly within collagen fibers and resulted in the higher ultimate tensile stress. Alternatively, it was obvious that ultimate tensile stress for the fresh tissues after degradation was extremely low and almost could not be determined due to the extensive degradation. In contrast, as a result of fixation with glutaraldehyde or ADA, the ultimate tensile stress of fixed tissues did not decreased sharply after enzymatic degradation. Meanwhile, Fig. 3 also illustrated that the decline in mechanical strength for ADA-fixed tissues was more notable than that of glutaraldehyde-fixed tissues. This result was consistent with the previous result on the weight loss during degradation process, which means the stronger resistance against enzymatic degradation for glutaraldehyde-fixed tissues, compared to ADA-fixed tissues.

3.4. Histological examination

Histological examination mainly showed the total framework of the examined biological tissues. Fig. 4 presents the photomicrographs of the fresh, glutaraldehyde-fixed and ADA-fixed tissues stained with hematoxylin and eosin, before and after degradation in a collagenase solution for 24 h. After collagenase degradation, it was found that the fresh tissues were obviously chaotic and almost disintegrated into pieces, which indicated the extensive degradation effect by collagenase. In contrast, the microcosmic structures of both glutaraldehyde-fixed and ADA-fixed tissues remained intact.

On the other hand, as the critical elements for the structural integrity, the collagen fibers of naturally derived biological tissues are important for the adhesion and proliferation of cells. Hence, all studied tissues were also examined specially by Masson staining, in order to further compare the microscopic collagen fibers changes after collagenase degradation (Xu et al., 2012). Fig. 5 presents the photomicrographs of the fresh and fixed tissues stained with Masson, before and after enzymatic degradation. We can find that, the microcosmic structures of collagen fibers were all preserved well after degradation, as a result of fixation with glutaraldehyde or ADA [Fig. 5(b,b‘,c,c‘)]. In contrast, the collagen fibers of fresh tissues after collagenase degradation were degraded extensively [Fig. 5(a,a‘)]. This result was also fully consistent with the previous observation on hematoxylin and eosin staining.

3.5. Scanning electron microscopy (SEM)

To further compare the microscopic changes caused by enzymatic degradation at the higher magnification, SEM was performed to examine the surface morphology of each test tissue. As shown in Fig. 6, there was considerable fracture observed in the fresh tissues

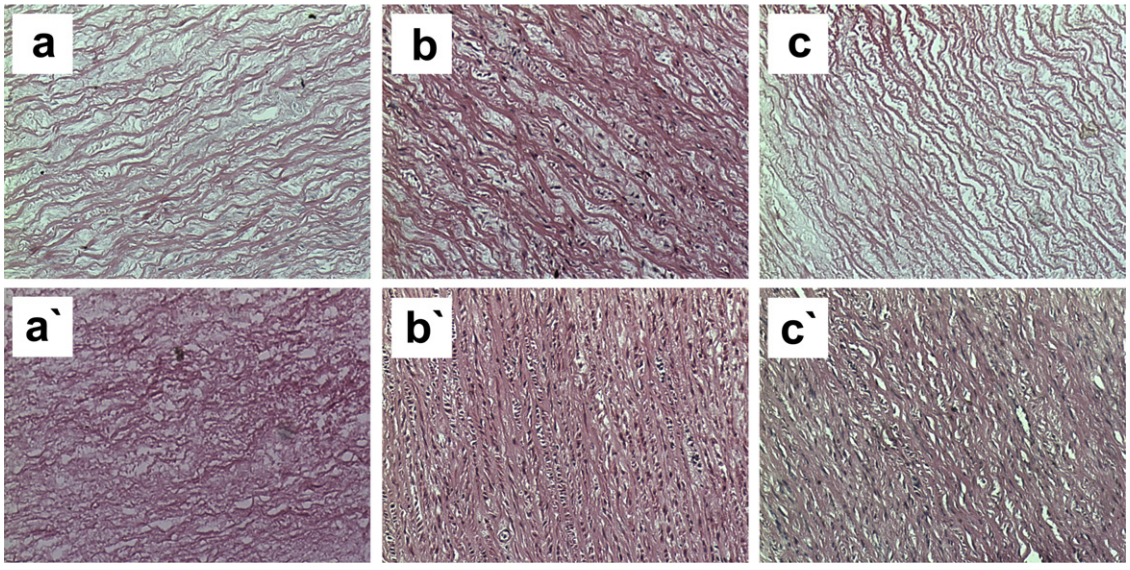

Fig. 4. Photomicrographs (stained with hematoxylin and eosin) of the (a) fresh tissues, (b) glutaraldehyde-fixed tissues, (c) ADA-fixed tissues before degradation; (a‘) fresh tissues, (b‘) glutaraldehyde-fixed tissues, (c‘) ADA-fixed tissues after degradation.

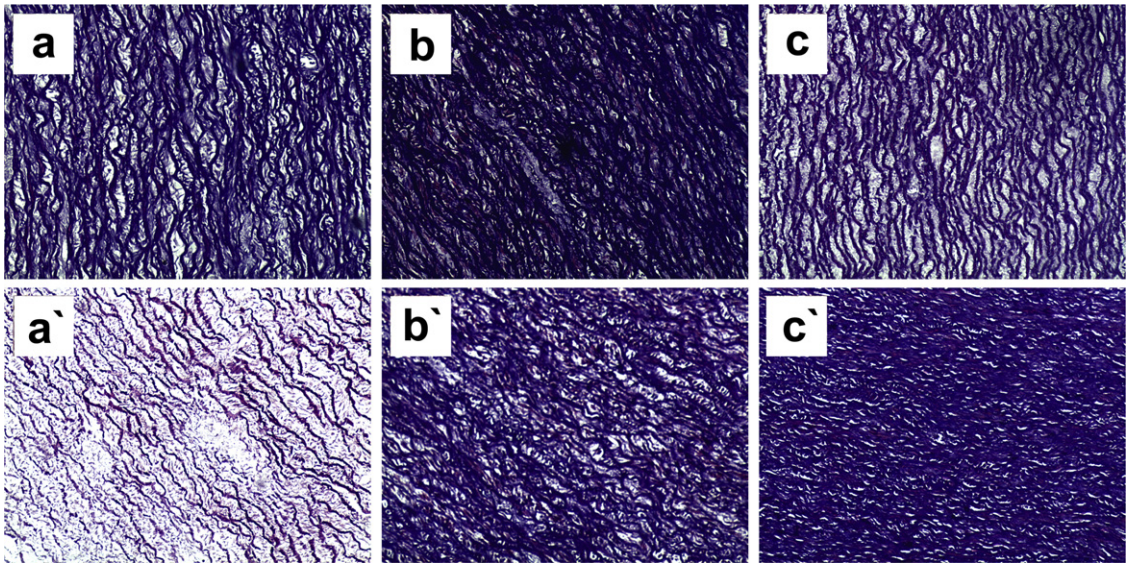

Fig. 5. Photomicrographs (stained with Masson) of the (a) fresh tissues, (b) glutaraldehyde-fixed tissues, (c) ADA-fixed tissues before degradation; (a') fresh tissues, (b') glutaraldehyde-fixed tissues, (c') ADA-fixed tissues after degradation.

after collagenase digestion [Fig. 6(a,a')], which indicated the extensive degradation effect by collagenase. However, as compared to their counterparts before digestion, the glutaraldehyde-fixed and ADA-fixed tissues after collagenase digestion did not show significant degradation. It was noted that the microscopic structure was clearer and more orderly, and almost no fracture was observed in fixed tissues [Fig. 6(b,b',c,c')]. Similar results were also observed in the previous morphologic observation (Fig. 1) and histological examination (Figs. 4 and 5).

3.6. The degradation mechanism of the fixed tissues

Based upon these qualitative results in the above morphological observations, histological examination and SEM observations, one may conclude that the glutaraldehyde or ADA fixation process could enhance the resistance against enzymatic degradation of biological tissues effectively, which might mean a protection from immediate degradation in vivo after implantation. This remarkable resistance against degradation observed in the fixed tissues probably resulted from structural changes in collagen, i.e. the cleavage sites of collagen being hidden or altered by the reaction of the free amino groups in the fixation process (Sung et al., 2000). Generally, collagenase has a unique site of attack in the collagen molecule, namely, the primary sequence of the amino acids leucine and glycine, constituting a 'weak' area for extensive proteolytic attack. However, the many intermolecular crosslinks after fixation by multiple functional aldehyde groups could make the penetration of enzymes into those attack sites more difficult, resulting in the inhibition of enzyme–substrate interaction (Vizárová et al., 1995).

Alternatively, the quantitative results in the weight loss testing and mechanical testing suggested that the resistance against enzymatic degradation of ADA-fixed tissue was not stronger than that of glutaraldehyde-fixed tissue. This difference in resistance between the two crosslinking reagents may be caused by their

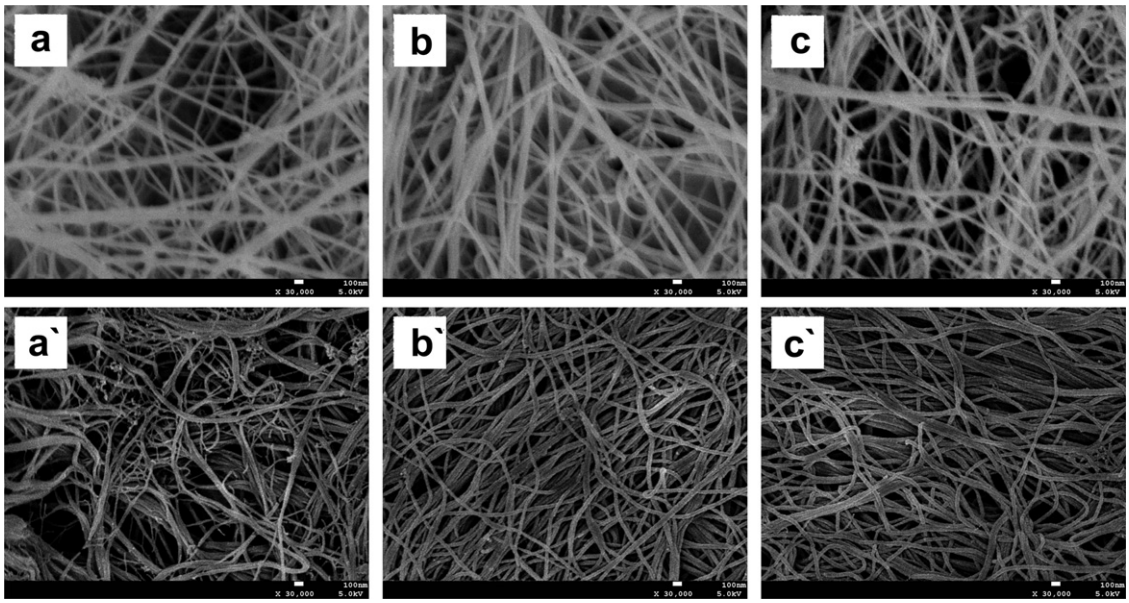

Fig. 6. Scanning electron micrographs of the (a) fresh tissues, (b) glutaraldehyde-fixed tissues, (c) ADA-fixed tissues before degradation; (a') fresh tissues, (b') glutaraldehyde-fixed tissues, (c') ADA-fixed tissues after degradation.

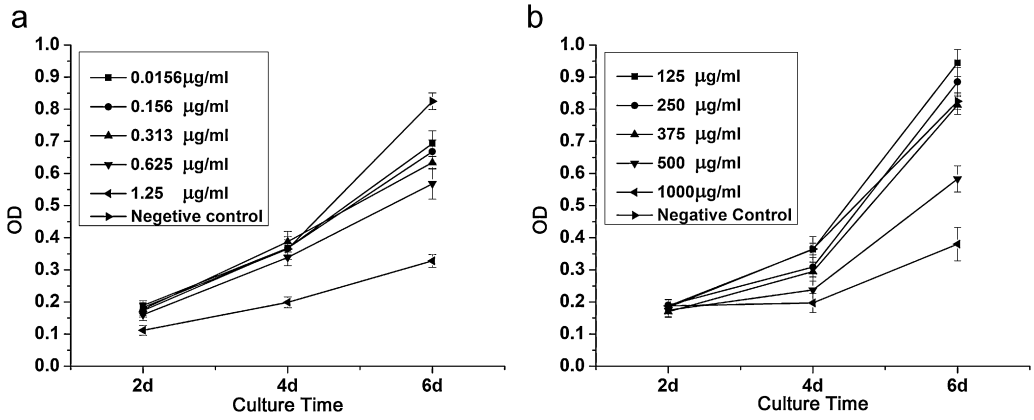

Fig. 7. The effect of glutaraldehyde and ADA on the proliferation of L929 fibroblasts. (a) glutaraldehyde; (b) ADA.

difference in chemical structure and potential degradability. As mentioned previously, the molecular weight of ADA (about 10000) is much larger than that of glutaraldehyde (about 100), which might make the diffusion of ADA into the collagen molecule more difficult than glutaraldehyde (Xu et al., 2013). This different diffusion effect between glutaraldehyde and ADA could result in different crosslinking degree. Therefore, the tissues fixed by glutaraldehyde have the more significant inhibition of enzyme–collagen interaction, and thus result to the stronger resistance against enzymatic degradation. On the other hand, the biodegradability of ADA itself may also result in the degradation of the fixed tissues. It has been reported that, when alginate is oxidized to ADA by periodate, the carbon-carbon bond of the cis-diol group in the uronate residue is cleaved, and the aldehyde groups of oxidized hexuronic-acid residues spontaneously form six-membered hemiacetal rings with the closest hydroxyl groups on the two adjacent, unoxidized sugar residues in the chains. This alters the conformation of the uronate residues to an open-chain adduct and infers a free rotation about the three bonds adjoining C-4, C-5, the original ring-oxygen atom, and C-1 of the oxidized hexuronic-acid residues. This creates hydrolytically labile bonds in the polysaccharide (Boontheekul, Kong, & Mooney, 2005; Gao, Liu, Chen, & Zhang, 2009). As a consequence, we should assume that the biodegradability of ADA might make the ADA-fixed tissue fully biodegradable with time in the body.

3.7. *The tissue engineering applications of the ADA-fixed tissues*

Besides the degradation of the ADA-fixed tissues, we further studied the effect of crosslinking reagents on the cells proliferation, because the biocompatibility is also very important for the applications in tissue engineering. Fig. 7 illustrates the effect of varying concentrations of glutaraldehyde and ADA on the proliferation of L929 fibroblast. It was noted that, compared to the negative control, glutaraldehyde exhibited an inhibition effect on cell proliferation even at the extremely low concentration of 0.0156 μg/ml. Meanwhile, glutaraldehyde exhibited an obvious cytotoxicity when its concentration was more than 0.625 μg/ml. In contrast, the similar inhibition effect on cell proliferation of ADA crosslinking reagent could be observed until the significantly higher concentration of 500 μg/ml. This result indicated that the cytotoxic effect of ADA was nearly 1000 times lower than that of glutaraldehyde. In addition, our previous study suggested that the ADA-fixed tissues also demonstrated an excellent cytocompatibility. This large difference in the effect on cells proliferation between the glutaraldehyde and ADA may be caused by their different sources and chemical structures. Glutaraldehyde is typically synthetic crosslinking reagent while ADA is derived from alginate, a naturally occurring polysaccharide in possession of high biocompatibility. Additionally, the molecular weight of ADA (about 10000) is much larger than that of glutaraldehyde (about 100), which might make the diffusion of ADA into the cells more difficult than glutaraldehyde. Therefore, ADA is more difficult to react with proteins or polysaccharides on and inside the cells, and thus result to less cell death than that of glutaraldehyde (Xu et al., 2013).

Moreover, in the study of effect on the cells proliferation, of note is that L929 fibroblasts proliferated faster in medium supplemented with 125–250 μg/ml ADA than in the negative control group, which suggested that ADA even had a stimulatory effect on cell proliferation at a low concentration. This unique stimulatory effect on cell proliferation could be great important for the tissue regeneration in tissue engineering construction.

Tissue loss or end-stage organ failure resulting from an injury or a disease has been a major concern in healthcare for many years, as they are costly and devastating problems (Langer & Vacanti, 1993). In 1987, tissue engineering was formally coined for the first time and then was considered as a potential alternative to tissue or organ transplantation (Chapekar, 2000; Lanza, Langer, & Vacanti, 2000). The objective of tissue engineering is to regenerate natural tissues from living cells to repair or replace failing tissues and organs. Generally, the process of tissue engineering construction involves the seeding of cells onto a scaffold. This whole is cultured in vitro and finally implanted into the body as prosthesis when matured. The natural tissue regeneration processes then take place, blood vessels infiltrate the structure and the scaffold eventually degrades, leaving behind a newly formed tissue in place (Liu, Xia, & Czernuszka, 2007; Rabkin & Schoen, 2002). It is known that the tissue engineering scaffold mainly acts as a temporary support for the cells to attach, proliferate and differentiate. Degradability is often a critical property of scaffolds utilized in tissue engineering because the second surgery to remove them (such as a heart patch) would be averted if the substrate could be removed by the physiological system of the host body (Chen et al., 2008). Moreover, in many applications, the tissue engineering constructs cannot be removed, and the non-degradable biomaterials would act as barriers to new tissue ingrowth and blood flow. On the contrary, an entirely degradable scaffold can be progressively relieved of function and replaced by new tissue as degradation progress. Complete degradation would alleviate many concerns about long-term implant biocompatibility (Slaughter et al., 2009).

In our study, the degradability of biological tissues fixed by ADA was evaluated by comparing the changes before and after enzymatic degradation. The results obtained in the study suggested that not only the ADA-fixed tissues could degrade gradually over time, but also the ADA crosslinking reagent had a stimulatory effect on cell proliferation at an appropriate concentration. Alternatively,

one of the most important challenges in tissue engineering construction is to design a scaffold material that could biodegrade in a rate matching the growth kinetics of a specific tissue at a specific anatomic position (Chen et al., 2008). It was also reported that the degradation rates of ADA could be controlled by changing the degree of oxidation (Gao et al., 2009). This type of controllable degradation may have significant advantages for biomedical applications. Moreover, for the biological tissue fixation, the different crosslinking degree could also result in the different tissue degradation rate and its tissue regeneration pattern (Liang, Chang, Hsu, Lee, & Sung, 2004). Therefore, these multiple controllable factors may lead to biodegradation in vivo at rates appropriate to tissue regeneration, providing an ideal solution to the challenge mentioned above.

As a consequence of these results, we assume that the ADA-fixed tissue might be a successful example of the applications in the tissue engineering. That is to say, when implanted in humans, the ADA-fixed tissue averts the immediate degradation at first. Subsequently, the ADA begins to induce the tissue regeneration gradually, mainly due to the excellent cytocompatibility. Meanwhile, the ADA-fixed tissue also begins to degrade over time, as a result of the good degradability. Ultimately, the ADA-fixed tissue degrades completely, leaving behind a newly formed tissue in place. On the contrary, the tissues fixed by those traditional synthetic crosslinking reagents might produce significant cytotoxic effect and act as barriers to new tissue ingrowth and blood flow when used in the tissue engineering applications.

4. Conclusion

In conclusion, this study mainly demonstrated the enzymatic degradation of the biological tissues fixed by ADA, a promising crosslinking agent in possession of excellent cytocompatibility. The result suggested that, the ADA fixation could enhance the resistance against enzymatic degradation of biological tissues effectively. Meanwhile, the ADA-fixed tissues could also degrade gradually over time, compared to glutaraldehyde-fixed tissues. In addition, the ADA crosslinking reagent itself had a stimulatory effect on cell proliferation when at an appropriate concentration. The results obtained in this study in vitro demonstrate that ADA fixation might provide a successful example of the biodegradable scaffold materials in tissue engineering, mainly due to its degradability and cytocompatibility. Moreover, our further study will pay attention to the biocompatibility and regeneration of the ADA-fixed tissues in vivo.

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