Journal of Applied Polymer Science

Evaluation of alginate dialdehyde as a suitable crosslinker on modifying porcine acellular dermal matrix: The aggregation of collagenous fibers

Shichen Zhu,[1,2] Zhipeng Gu,[3] Yang Hu,[1,2] Weihua Dan,[4] Shanbai Xiong[1,2]

[1]College of Food Science and Technology and MOE Key Laboratory of Environment Correlative Dietology, Huazhong Agricultural University, No.1 Shizishan Street, Hongshan District, Wuhan, Hubei 430070, China

[2]The Sub Center (Wuhan) of National Technology and R&D of Staple Freshwater Fish Processing, Wuhan 430070, China

[3]Department of Biomedical Engineering, School of Engineering, Sun Yat-sen University, Xiaoguwei Island, Panyu District, Guangzhou 510006, China

[4]Department of Biomass Chemistry and Engineering, Sichuan University, No.24 South Section 1, Yihuan Road, Chengdu, Sichuan 610065, China

Shichen Zhu and Zhipeng Gu contributed equally to this work.

Correspondence to: Y. Hu (E-mail: huyang@mail.hzau.edu.cn)

ABSTRACT: Porcine acellular dermal matrix (PADM) has been investigated widely as a natural biomaterial. However, the success of PADM has been limited by insufficient stability and poor physicochemical properties. In our work, alginate dialdehyde (ADA) with various oxidation degrees (OD = 25%, 45%, and 65%) were explored to modify PADM, while glutaraldehyde (GA), dehydrathermal treatment (DHT), and carbodiimide (EDC) were used as the control. The efficacy of ADA on modifying PADM increased along with the rising of oxidation degree. The ADA (OD = 65% and 45%) groups showed better mechanical and thermal stability, crosslink density, and resistance to enzymatic degradation than ADA (OD = 25%) and DHT + EDC group. Meanwhile, the structure of PADM crosslinked by ADA (OD = 45% and 65%) were maintained largely. Further, ADA (OD = 45%) group revealed better cytocompatibility than DHT + EDC, ADA (OD = 65%) and GA group. Considering the balance of cytocompatibility and physicochemical behavior, ADA (OD = 45%) was more suitable as a natural derived crosslinker to modify PADM in tissue engineering. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43550.

KEYWORDS: biocompatibility; biomaterials; biomedical applications

Received 11 October 2015; accepted 18 February 2016

DOI: 10.1002/app.43550

INTRODUCTION

Recently, porcine acellular dermal matrix (PADM), which is mainly composed of type I collagen has gained the most attention of researchers in the field of tissue engineering and regeneration due to its excellent biological achievements.[1–3] As a biological scaffold, PADM is obviously biocompatible and not only provide mechanical support serving as an ideal three-dimensional (3D) architecture for host cells to grow and metabolize, but also facilitate cell-scaffold interactions, which actively influence cellular responses, for instance, cell proliferation, and differentiation.[4,5] Accordingly, PADM has been extensively applied as substrates for skin repairing, as implants for breast shaping, and as scaffolds for dura matter, bone, and soft connective tissue remodeling.[3,6] Despite many of the superiorities of PADM as sketched above, the relatively weak enzyme-resistance property of PADM may limit its use as biological scaffolds which need to be faced up to. Hence, PADM is often chemical or physical modified to adjust its physicochemical properties, especially the enzymatic degradation rate to meet the clinical requirements, with the methods of UV-light irradiation, dehydrathermal treatment (DHT), and synthetic crosslinking reagents (carbodiimide (EDC), glutaraldehyde (GA), and polyepoxy compound).[1,2] However, the UV-light irradiation treatment confines to the surface modification of PADM, the treatment by DHT makes little contribution to the physicochemical property of PADM and the cytotoxic nature of synthetic crosslinking agents i.e., EDC, GA, and polyepoxy compound reflected in the process of modifying PADM may prohibit their utilization in the field of tissue engineering.[4] To overcome the aforementioned cytotoxic effect of synthetic crosslinking agents and the low efficacy of physical crosslinking treatments, a natural derived crosslinker, alginate dialdehyde (ADA) was developed in our previous work.[7]

Alginate (ALG), ubiquitously found in brown algae, is a biocompatible, nonimmunogenic, and biodegradable natural negatively charged polysaccharide, of which the typical structure is a

block copolymer consists of two sterically different repeating units, (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers.[8–12] It is worthy to note that ALG could be oxidized to form a natural derived crosslinker (alginate dialdehyde, ADA) with multiple functional aldehyde groups, of which the active group could react with the free amino groups the same as glutaraldehyde.[7,13–16] In our previous work, the interaction between ADA and chitosan plus silk was investigated preliminarily, the results indicated that ADA stabilizes chitosan as a crosslinker and preserves its typical structure, which may pave the way for developing ADA as a natural derived crosslinker for chitosan in tissue engineering.[7]

However, unlike chitosan or silk,[7] PADM is the hierarchical aggregate of collagen molecules, and the physicochemical behavior of PADM modified by alginate dialdehyde, especially the mechanical property, thermal stability, and enzyme-resistance property of PADM remains largely unknown, which is also quite important to develop ADA as a crosslinker for PADM in future. Hence, it is meaningful to further evaluate alginate dialdehyde as a suitable crosslinker on modifying porcine acellular dermal matrix. In this work, we have investigated the corresponding physicochemical behaviours, i.e. mechanical property (tensile strength and elongation at break), structure integrity, thermal stability, enzymatic resistance ability, and biocompatibility of PADM modified by ADA compared to normal crosslinking reagent (glutaraldehyde, carbodiimide, and dehydrothermal treatment). Our aim is to evaluate the feasibility of ADA as a natural derived crosslinker for PADM scaffold manufacturing in tissue engineering.

EXPERIMENTAL

Materials

Sodium alginate (viscosity: 495 cps at 25 °C) was purchased from Qingdao Jingyan Biotechnology Co. Ltd. (China). Glutaraldehyde and carbodiimide were obtained from Sigma-Aldrich (St. Louis, MO, USA). PADM with the average size of 7 cm (length) × 4 cm (width) was manufactured according to our previous work.[1] Alginate dialdehyde with different oxidation degrees (OD, ~25%, 45%, and 65%) were obtained via oxidation process by using sodium periodate with different dosages according to our previously reported method.[7] Other reagents were used as received.

Crosslinking Process

For alginate dialdehyde (ADA) crosslinking process, the mass ratio of ADA was controlled in the range of 10%, and the weighted ADA with different oxidation degrees (~25%, 45%, and 65%) were respectively dissolved in phosphate buffered saline (PBS, pH 7.4, 10 m*M*/L) to prepare saturated ADA solutions with the ADA concentration of ~20% by weight approximately. Subsequently, PADM with the size of 7 cm (length) × 4 cm (width) was immersed into the saturated ADA solution for 48 h at room temperature (21 ± 1 °C) after wetting with deionized water. Excess ADA was rinsed from the matrix using 10 m*M* PBS (pH 7.4) twice for 30 min and washed by distilled water three times, each time for 30 min. Then, PADM samples were frozen at −50 °C for 5 h and lyophilized at low chamber vacuum (0.05 bar) as described previously.[1,16] Meanwhile, PADM was incubated in glutaraldehyde (GA) solution with the concentration of 0.5% for 48 h at room temperature (21 ± 1 °C) and proceeded the same washing and lyophilization process as described above to obtain the GA crosslinked PADM group as the control. Besides, PADM crosslinked by dehydrathermal (110 °C) and carbodiimide (15 m*M*/g PADM) composite modification was carried out according to our previous work to gain the DHT + EDC crosslinked PADM group as the control.[1,7]

Crosslink Density

The crosslink density was detected by a slightly modified ninhydrin assay according to our previous work, since the amount of free amino groups after heating with ninhydrin is proportional to the optical absorbance of the solution.[1] Then, the crosslink density was calculated by the following formula:

$$\text{Crosslink density}(\%) = [(NH_{2\text{before}}) - (NH_{2\text{after}})]/(NH_{2\text{before}}) \times 100 \quad (1)$$

where $NH_{2\text{before}}$ is the amount of free amino groups in PADM sample before crosslinking and $NH_{2\text{after}}$ is the amount of free amino groups in the sample after crosslinking. The crosslink values were recorded as mean ± standard deviation (SD, n = 5).

Histologically Observation

PADM specimens crosslinked by ADA, GA, and DHT + EDC were firstly cut into pieces with the size of 0.5 mm (length) × 0.5 mm (width), subsequently fixed in 10% formaldehyde, ice embedded, sectioned, and stained with hematoxylin–eosin (HE) assay.[5]

In Vitro Enzymatic Degradation

PADM specimen resistance to degradation *in vitro* was assessed through exposure to bacterial collagenase type I (Sigma-Aldrich, America). Dry PADM specimens were cut into 10 mm diameter circles (n = 5 per group), weighted (W_1), and incubated in collagenase type I solution (1 U/mL, 3 mL/mg PADM specimen) at 37 °C for 7 days. PADM specimens were removed from the medium at day 1, 2, 4, and 7, then rinsed by 10 m*M* phosphate buffered saline (PBS, pH 7.4) followed by distilled water. Subsequently, the specimens were lyophilized and weighted (W_2). Eventually, the degradation rate or weight loss percentage could be calculated by the following formula:

$$\%\text{Degradation} = [(W_1 - W_2)/W_1] \times 100 \quad (2)$$

Mechanical Test

Tensile strength and elongation at break testing were used to determine the effect of ADA on the mechanical properties of PADM. PADM specimens were cut into rectangular strips (20 mm × 4 mm) and pre-hydrated in phosphate buffered saline (PBS, pH 7.4) for 1 h prior to testing.[1] In addition, PADM specimens' mechanics were carried out by using a mechanical testing machine (AI-7000S, Gotech, China) with a strain rate of 5 mm/min. Each test was conducted on five samples and the results were expressed as mean ± standard deviation.

Microstructure and Porosity

A scanning electron microscopy (SEM, Hatachi Model S520, Japan) was used to characterize the morphology of crosslinked PADM both before and after enzymatic degradation *in vitro*.

Table I. The Effect of Different Crosslinking Methods on the Mechanical Properties of PADM Scaffold [(a) No Crosslinking, (b) GA, (c) ADA (OD = 25%), (d) ADA (OD = 45%), (e) ADA (OD = 65%), and (f) DHT + EDC Group].

	Before degradation		After degradation (7 days)		
	Tensile strength (MPa)	Elongation at break (%)	Tensile strength (MPa)	Elongation at break (%)	Crosslink density (%)
A	1.8 ± 0.2	21.5 ± 3.3	—	—	0
B	8.2 ± 0.3[a]	13.5 ± 2.9[a]	5.8 ± 0.2	9.1 ± 1.3	95.2 ± 3.3
C	5.2 ± 0.2[a]	18.1 ± 3.1	2.1 ± 0.1[b]	7.2 ± 0.9[b]	63.4 ± 3.2[b]
D	7.9 ± 0.4[a]	14.7 ± 2.1[a]	5.1 ± 0.3	8.4 ± 1.1	91.5 ± 4.5
E	7.6 ± 0.3[a]	14.2 ± 1.9[a]	5.5 ± 0.4	8.8 ± 0.8	93.2 ± 3.4
F	7.1 ± 0.6[a]	15.3 ± 2.2[a]	3.2 ± 0.2[b]	7.0 ± 0.8[b]	77.3 ± 5.9[b]

[a] Compared with (a), $P < 0.05$.
[b] Compared with (b), (d), and (e), $P < 0.05$.

PADM specimens were removed from the incubating medium and rinsed using 10 m*M* phosphate buffered saline (PBS, pH 7.4) twice followed by washing with distilled water three times. Then, lyophilized PADM specimens were sputter coated with aurum and imaged at an accelerating voltage of 5 kV, while no-crosslinking group as the control. Further, Image analysis software (ImageJ, Wayne Rasband) was applied to determine the mean pore diameter and distribution within the PADM scaffold. In addition, the total number of pores analyzed for each sample was 500. The porosities of PADM scaffolds were performed according to our previous methods by using the Archimedes principle, and the values were given as mean ± standard deviation (n = 8).[1]

TG Measurements

Thermal weight loss of PADM scaffolds was detected by thermogravimetric analyzer (Netzsch TG 209F1, Germany) from 35 °C to 800 °C with the heating rate of 20 °C/min.

Cell Culture and Cell Proliferation Assay

The biocompatibility of PADM crosslinked by ADA, GA, and DHT + EDC were assessed using L929 fibroblasts with the method of 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazoliumbromide (MTT) assay.[7] Briefly, PADM specimens were firstly cut into pieces with the size of 10 mm (length) × 10 mm (width) and sterilized by ethylene oxide vapour.[1,7,16] Then PADM specimens were incubated in 24-well cell culture plates seeded with 5×10^4 cells/mL in 500 μL medium at 37 °C with 5% CO_2 for 1, 3, and 5 days, while the medium was changed every 2 days. At each time point, 20 μL 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazoliumbromide (MTT) was added into the culture medium and incubated for 4 h at 37 °C. Subsequently, the medium was removed and 1.5 mL dimethylsulfoxide (DMSO) was added into the culture plate to dissolve formed formazan with vibration for 10 min. Then the optical density value of each well was measured with a microplate reader (Model550, Bio Rad Corp., America) at 492 nm.

Confocal Laser Scanning Microscopy Observation

PADM crosslinked by ADA, GA, and DHT + EDC were seeded with L929 fibroblasts at a density of 2.5×10^4 cells/well, and cultured at 37 °C with 5% CO_2 for 3 days. Then, PADM specimens were rinsed by phosphate buffered saline (PBS, pH 7.4) gently three times and firstly stained by fluorescein isothiocyanate (FITC) with the concentration of 10 μg/mL for 2 h at 37 °C. Subsequently, PADM specimens were washed with PBS three times and fixed by 4% paraformaldehyde in PBS for half an hour at room temperature. After gently washing with PBS twice, PADM specimens were stained with 10 μg/mL 4,6-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Finally, the samples were rinsed with PBS and then mounted by glycerol/PBS and observed using a confocal laser scanning microscope (Leica TCS SP II, Leica, Germany).

Statistical Analysis

All results were expressed as mean ± SD (standard deviation). Statistically significant differences ($P < 0.05$) between the testing groups were measured by one-way analysis of variance (ANOVA) on SPSS 19.0.

RESULTS AND DISCUSSION

Mechanical Properties and Crosslink Density Analysis

Mechanical properties, such as the tensile strength and elongation at break are usually considered crucial for various biomaterials, which could provide the biomaterials with sufficient tension and strength in clinical application especially when it is used as wound dressing at the joints, ankles or elbows.[1,17,18] Table I shows us the significant impacts of various crosslinking methods on the mechanical properties of PADM. ADA with different oxidation degrees (~25%, 45%, and 65%), GA, and DHT+EDC did promote the tensile strength of PADM to some extent. Before crosslinking, the tensile strength of PADM is 1.8 MPa approximately, while after crosslinking the tensile strength reaches up to ~8.2 MPa for GA group, ~7.1 MPa for DHT + EDC group, ~5.2 MPa, 7.9 MPa, and 7.6 MPa for ADA group with different oxidation degrees (OD = 25%, 45%, and 65%) respectively. The results illustrate that crosslinkers with suitable amount of aldehyde groups show better modification effect on promoting the tensile strength of PADM than the synergistic effect of DHT and EDC crosslinking.

As revealed in the scheme illustration (Figure 1), it is worthy to note that PADM is not the simple stack of collagen molecules, but the accurate assembling of collagen triple-helixes.

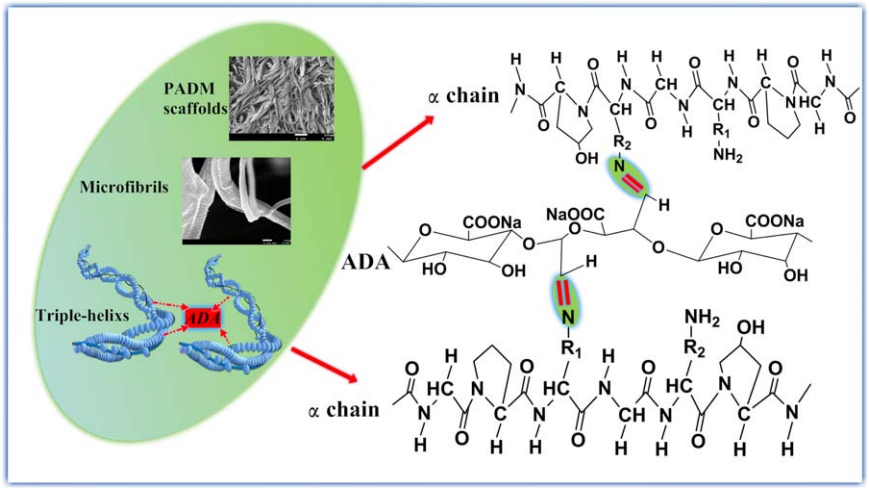

Figure 1. Schematic diagram showing the possible interactions between PADM and alginate dialdehyde. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Firstly, the three α-chains twist together into a unique triple helical molecule (collagen molecule with ~300 nm in length and ~1.5 nm in diameter). Then the typical quarter staggered arrangement of collagen molecules constitutes collagen microfibrils with ~40 nm in diameter and fibrils with about 100–200 nm in diameter. Further, the fibrils assemble into collagen fibers to form the basic framework of PADM.[19] As reported in the literatures, there are so many potential active groups within PADM, for instance the free amino groups (–NH_2), free carboxyl groups (–COOH), and hydroxyl groups (–OH) of collagen side chains within PADM, and amide bond (–HN–CO–) of collagen backbones within PADM.[20,21] For carbodiimide crosslinking, firstly water could act as a nucleophile which could lead the hydrolysis of the O-acylisourea group to give the substituted urea and the starting carboxylic acid group. Secondly, a more stable *N*-acylurea group forms by the rearrangement of highly reactive O–acylisourea group, of which the *N*-acyl shift is independent of the presence of a nucleophile. Further, NHS could help convert the O-acylisourea group into NHS-activated carboxylic acid group that is more stable than the O-acylisourea group itself in acidic condition. After that, the carbodiimide molecule can be leached out by sufficient wash, only with stable amide bond left between adjacent free carboxyl groups (–COOH) and amino groups (–NH_2) of collagen side chains within PADM.[22] While for dehydrathermal treatment, it has been demonstrated that DHT crosslinking could promote the mechanical properties of PADM to some extent also by the amide bond forming, however the crosslinking effect can be restricted by the amount limitation of amino acid resides in the active center (i.e., aspartic acid, glutamic acid, serine, threonine, arginine, and lysine residues).[23] Further, our previous work and other scientists reported that the synergistic effect did exist between carbodiimide and dehydrathermal treatment during the crosslinking procedure of PADM, hence we choose the optimized conditions to crosslink PADM as the control [dehydrathermal (110 °C) and carbodiimide (15 m*M*/g PADM)].[1,2,22] However, the final tensile strength results suggest that this kind of amide bond crosslinking is not so effective than that of aldehyde crosslinking. The main reason may be attributed to the different reaction mechanism of aldehyde crosslinking. Traditionally, GA is firstly used in the leather industry to improve the thermal stability of hide or skin, after that GA is begin to be applied to modify collagen in order to protect it from being degraded by collagenase.[24,25] Schiff's base type compounds are supposed to form between the aldehyde group of glutaraldehyde and the ε-NH_2 of lysine of collagen to achieve the final crosslinked effect, and the Schiff bases are stable under the crosslinking conditions and crosslinking involves the formation of glutaraldehyde polymers due to aldol condensation reactions.[26,27] Unlike carbodiimide, GA and ADA could also form inter-molecular crosslinking between separated collagen chains of PADM by the arm exhibition effect through glutaraldehyde polymers or ADA-based polymers so as to lead more crosslinking joints of Schiff bases. In addition, the crosslink density result further demonstrates the above hypothesis (the crosslink density for GA group and ADA group (OD = 45% and 65%) reached up to 90% above, much higher than DHT + EDC group). That may be why crosslinkers with suitable amount of aldehyde groups show better modification effect on promoting the tensile strength of PADM than the synergistic effect of DHT and EDC crosslinking.

Besides, to better reflect the crosslinking efficacy, we also performed the mechanical property testing of PADM after the degradation process by using collagenase (Table I). After 7 days' degradation, PADM without crosslinking degraded and its mechanical property could not be detected. PADM crosslinked by ADA with different oxidation degrees (~25%, 45%, and 65%) showed different mechanical property and crosslink density. The crosslink density for ADA (OD = 25%) group was ~63.4%, much lower than that for ADA (OD = 45%) and ADA (OD = 65%) group, about 91.5% and 93.2% respectively. As is illustrated in Figure 1, aldehyde group is the active group of ADA molecules, which could react with the free amino groups of PADM by Schiff-base crosslinking. Hence the amount of aldehyde groups mainly determines the crosslinking efficacy and

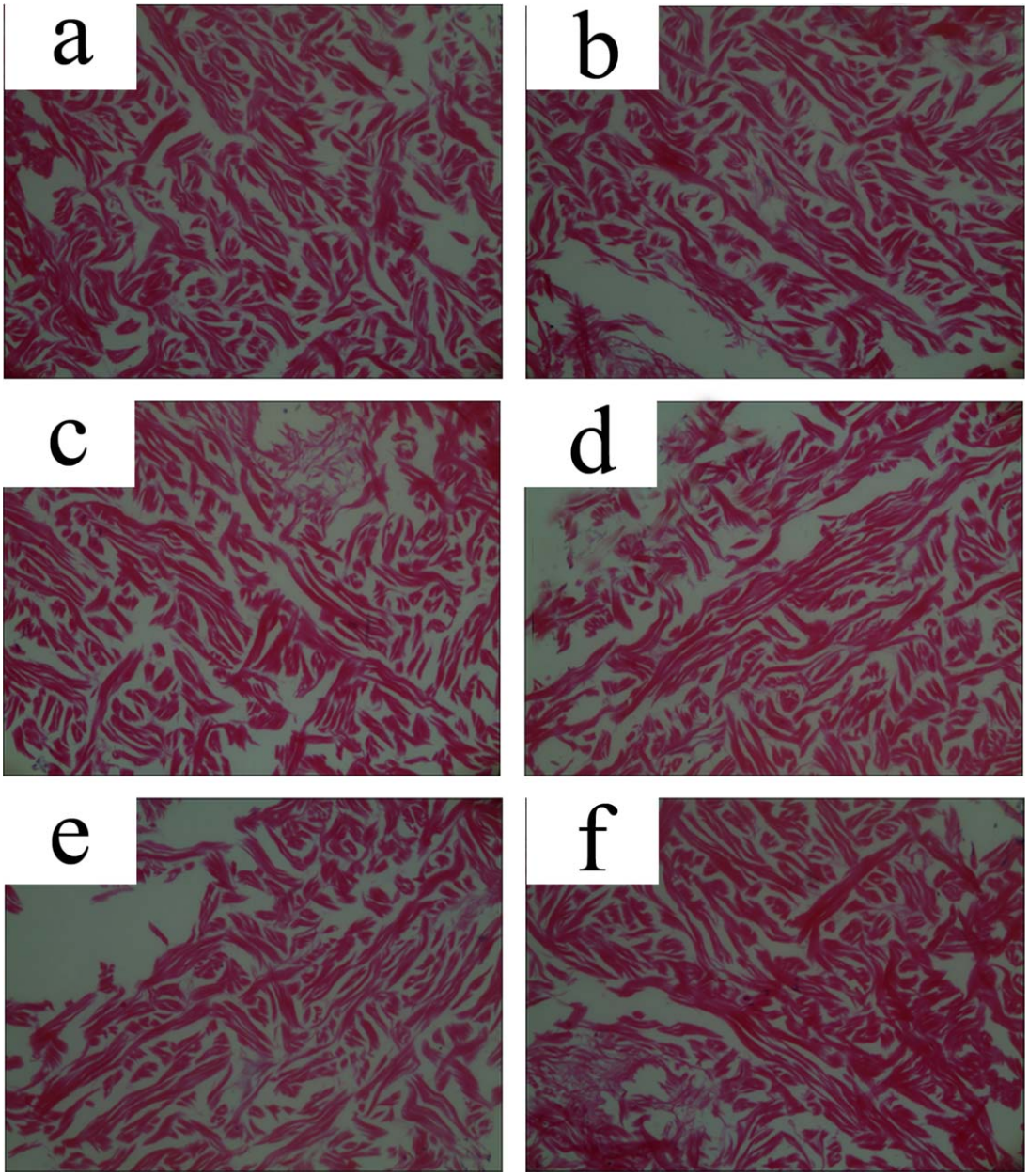

Figure 2. Histology shows the effects of various crosslinking methods on the distribution of skin collagen fibers within PADM scaffold [(a) No crosslinking group as the control, (b) GA group, (c) ADA (OD = 25%) group, (d) ADA (OD = 45%) group, (e) ADA (OD = 65%) group, and (f) DHT + EDC group, ×40]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

crosslink density.[20–22] Along with the oxidation degree increasing of ADA, the tensile strength of PADM increased accordingly from ~5.2 MPa for ADA (OD = 25%) group to ~7.9 MPa and ~7.6 MPa for ADA (OD = 45%) group and ADA (OD = 65%) group respectively. In addition, the crosslink density reached up to a relatively higher value (about 90%) which was much higher than that for DHT + EDC group (about 78%). One possible reason may be that no extra molecule introduced into PADM scaffold during DHT + EDC crosslinking process, which could restrict the crosslink effect between the amino and carboxyl groups within PADM far from each other.[22–24] In contrast, ADA with a certain chain could react with the free amino group by Schiff-base to form a more compact structure within PADM. However, it is not suitable to pursuit higher crosslink density by increasing the oxidation degree of ADA, since higher oxidation degree may cause the increasing of cytotoxicity.[27] Further, GA showed better crosslinking efficacy in tensile strength and resistance to enzymatic degradation. However the crosslink density of ADA (OD = 45%) group was getting close to that of GA group. Note that, both of the PADM specimens after crosslinking showed decreased elongation at break, and this phenomenon has been found in many references on collagenous scaffolds. The possible reason may be that rigid structure could formed after crosslinking, fibers within PADM tends to

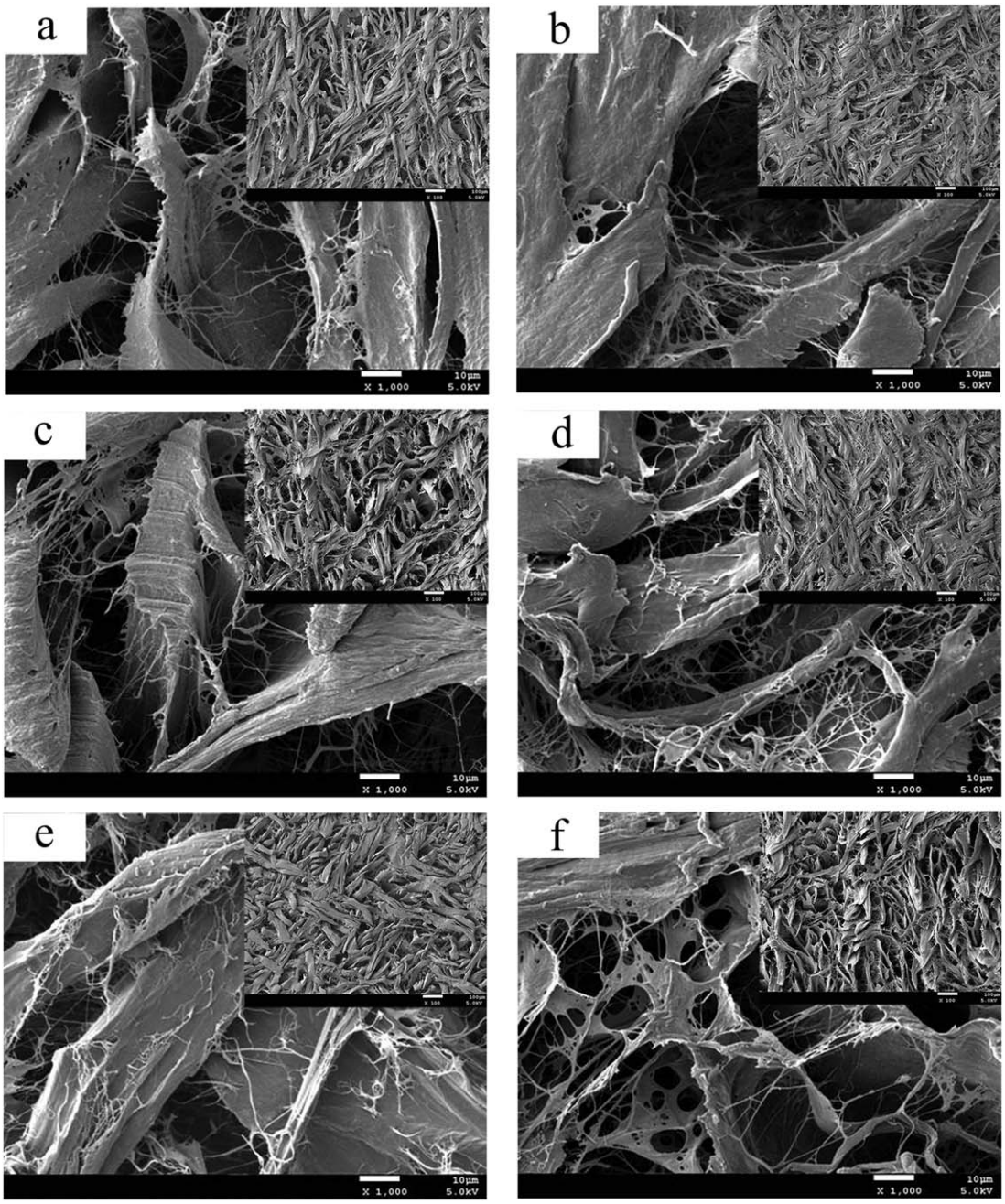

Figure 3. SEM images of PADM [(a) No crosslinking group as control, (b) GA group, (c) ADA (OD = 25%) group, (d) ADA (OD = 45%) group, (e) ADA (OD = 65%) group, and (f) DHT + EDC group]. The local magnifications of insets are denoted as "×100".

assemble directionally and hard to break which could cause stress concentration and brittle fracture during the mechanical testing.[1,25–27]

Morphological Analysis of PADM Before and After Crosslinking

As is well known, PADM is mainly composed of collagen fibers which could be stained red specifically by hematoxylin–eosin (HE) assay.[28] Hence, the cross-sectional structure of collagen fibers within PADM could be reflected by HE stained micrographs relatively as is shown in Figure 2(a–f). Generally, unlike collagen casting film, the collagen fibers within PADM reveals a three-dimensional (3D) architecture, which is favorable for host cell adhesion, growing into and proliferation.[29,30] Therefore, it has also been considered very important to maintain the 3D architecture of PADM during the crosslinking process. After crosslinking, the 3D hierarchical structure of PADM are retained to a large extent, and the corresponding pores are of irregular shape and sizes, but there is also a good pore inter-connectivity maintained in the scaffold microstructure of PADM as confirmed by the images [Figures 3 and 4(A)]. Generally, any crosslinking treatment may have some uncertain impacts on the

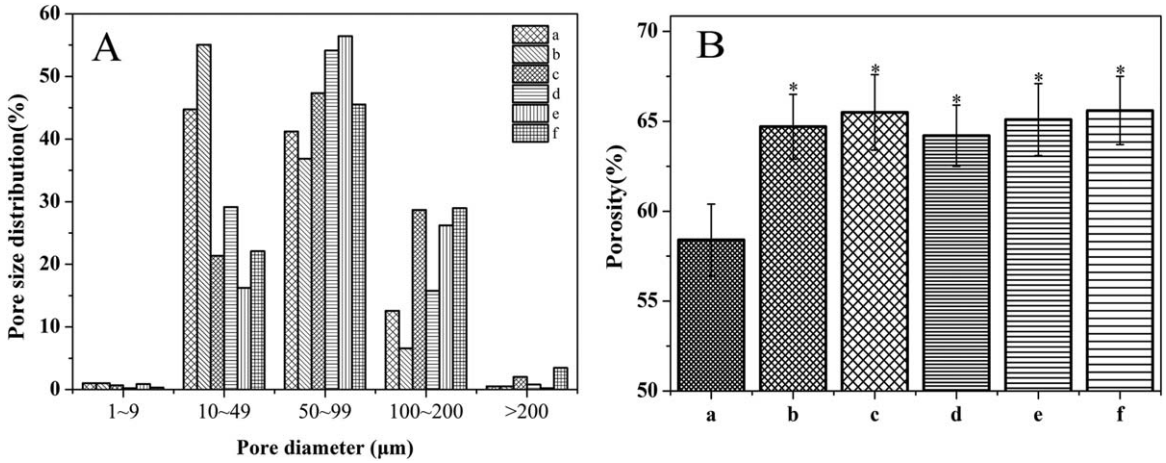

Figure 4. The pore size distribution (A) and porosity (B) of PADM before and after crosslinked [(a) No crosslinking group as control, (b) GA group, (c) ADA (OD = 25%) group, (d) ADA (OD = 45%) group, (e) ADA (OD = 65%) group, and (f) DHT + EDC group].

porosity and architecture of PADM scaffolds to some extent. Hence, we focused on the change of 3D architecture of PADM scaffolds after different crosslinking treatments. Practically, the HE images [Figure 2(a–f)] and SEM images [Figure 3(a–f)] illustrated the cross-sectional and surface view of these PADM scaffolds crosslinked differently, which indicated that the interconnected three-dimensional porous structure was retained to a large extent after crosslinking via GA, DHT + EDC, and ADA compared to no crosslinking group, although the porosity increased slightly from ~58% to ~66% [Figure 4(B)]. The possible reason why the porosity of PADM scaffolds increased was that PADM scaffolds after crosslinked had undergone the lyophilization process twice which could induce extra porosity within scaffolds.[6,24] Additionally, the structure integrity maintaining of PADM with interconnectivity has been demonstrated very important which allow not only the ingrowth of host cells and new tissues but also the free exchange of nutrients.[31–33] Finally, according to microstructure images, we may consider that PADM scaffold after ADA crosslinked remained its 3D hierarchical structure to a large extent and the structure integrity did not collapse.

In Vitro Enzymatic Degradation Analysis

PADM, as one of the promising natural biopolymers with interconnected 3D architecture has been widely investigated in tissue engineering owing to its chemotaxis to host cells and favors cellular attachment plus good biocompatibility and low antigenicity.[34–39] However, the relatively weak resistance to enzymatic degradation restricts its performance in clinical application. Hence, it is important to crosslink PADM with the method of chemical or physical crosslinking.

One of our present work aims to firstly investigate the efficacy and feasibility of ADA crosslinking on PADM during *in vitro* enzymatic degradation with compared to GA, EDC, and DHT crosslinking. Figure 5 illustrates the resistance ability to enzymatic degradation of PADM crosslinked by various methods (ADA, GA, and DHT + EDC) at different time intervals (day 1, 2, 4, and 7). As mentioned in Figure 1, PADM is mainly composed of type I collagen, of which the mass fraction reaches up to 90%.[1,38] Usually, collagen with integrity structure, i.e. a unique triple helical molecule comprises of two identical α_1(I) chains and a different α_2(I) chain, is hardly to be degraded in traditional protein enzymatic condition.[38] Therefore, application of collagenase is more convincing to detect the crosslinking efficacy of ADA on promoting PADM's resistance to enzymatic degradation. PADM without crosslinking shows lower resistance to collagenase degradation (Figure 5). After 7 days, the degradation rate for no crosslinking group (control group) reaches up to 80% approximately and the corresponding mechanical property is thoroughly lost and could not be detected (Table I) which is harmful for PADM in the clinical application. Note that the degradation rate of PADM crosslinked by ADA with different degrees of oxidation decrease sharply from ~30% for ADA (OD = 25%) group to ~17% for ADA (OD = 65%) group, respectively. Further, PADM crosslinked by ADA shows better resistance to collagenase degradation than DHT + EDC

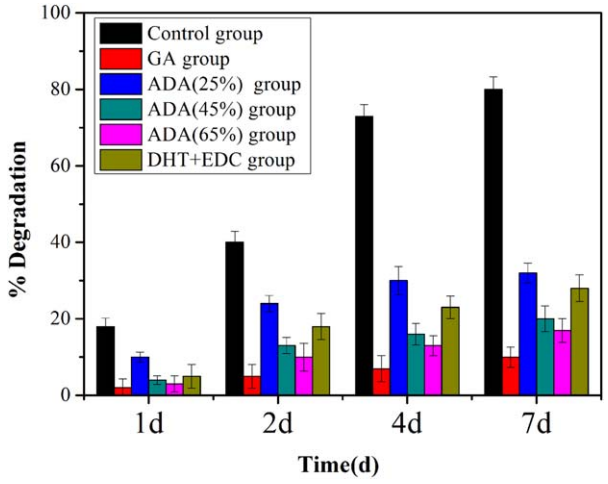

Figure 5. Effect of various crosslinking methods on the resistance to degradation of PADM scaffolds (No crosslinking group as the control, GA group, ADA (OD = 25%) group, ADA (OD = 45%) group, ADA (OD = 65%) group, and DHT + EDC group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

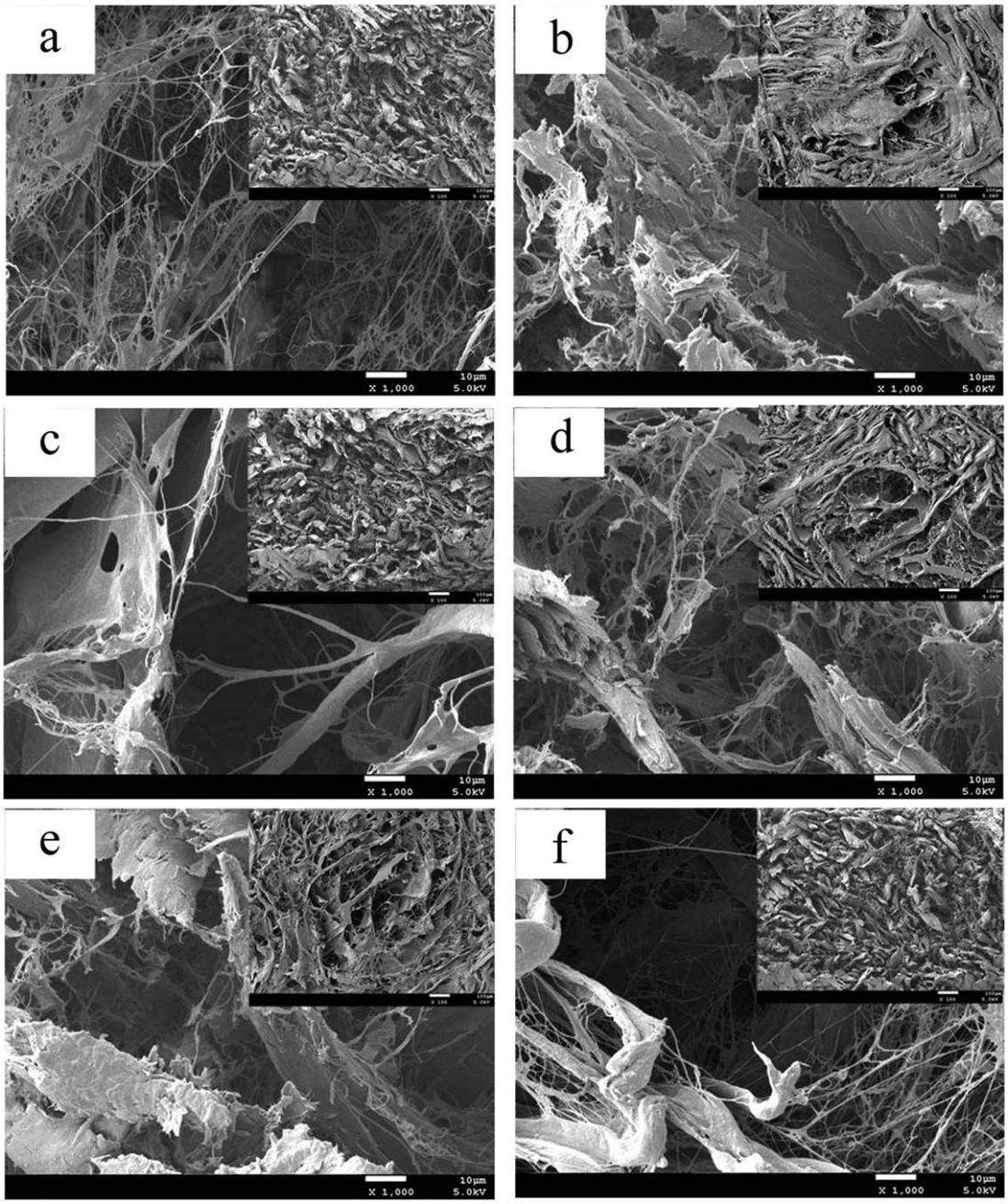

Figure 6. SEM micrographs of PADM scaffolds after degradation as a function of crosslinking method [(a) No crosslinking group as the control, (b) GA group, (c) ADA (OD = 25%) group, (d) ADA (OD = 45%) group, (e) ADA (OD = 65%) group, and (f) DHT + EDC group]. The local magnifications of insets are denoted as "×100".

group (~26% degraded) when the degree of oxidation of ADA reaches up to 45% (~19% degraded), approximately. Besides, GA as a previously widely used crosslinker did show better resistance to enzymatic degradation than ADA. However, the resistance to enzymatic degradation of PADM crosslinked by ADA (OD = 45% and 65%) appear to catch up with that for GA group. However, more investigation on *in vivo* degradation study should be performed in the future work because of the diversity between *ex situ* and *in situ* study.

Even more to the point, GA is not encouraged to be further used as the crosslinker for manufacturing biomaterials reported by many literatures due to the potential risk that glutaraldehyde crosslinked biomaterials implanted into human body for a period of time may cause tissue calcification and inflammatory response to some extent, which could affect the healing velocity[28–30] and ADA as a natural derived crosslinker could be developed as a potential crosslinker for PADM, while the stable properties and cytocompatibility of PADM crosslinked by ADA

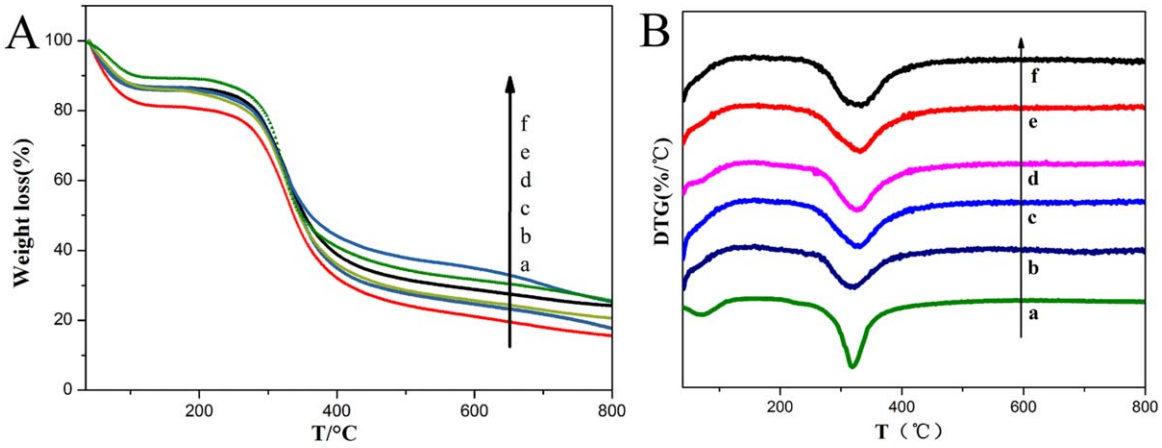

Figure 7. (A) Weight losses and (B) DTG thermograms of PADM scaffolds before and after crosslinking [(a) No crosslinking group as the control, (b) DHT + EDC group, (c) ADA (OD = 25%) group, (d) ADA (OD = 45%) group, (e) ADA (OD = 65%) group, and (f) GA group]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

should be further assessed and the results are expressed in the following section.

As is illustrated in many literatures,[2,3] PADM is mainly composed of extracellular matrices (collagen) with natural three-dimensional network structure after the removal of cells and cellular components. In addition, this unique hierarchical structure is more suitable than two-dimensional collagen membranes for host cell's adhesion, growing into and migration. Hence, it is important to evaluate the structure integrity of PADM after crosslinking and degradation, as is shown in Figure 6.

Figure 6 shows the morphological structure of PADM after degradation. Note that, after degradation for a period of 3 days, PADM without crosslinking begins to degrade by collagenase type I and the collagen fibers tend to be disintegrated, thus more tiny fibers are observed [Figure 6(a)]. In contrast, the crosslinking groups (GA group, ADA group, and DHT + EDC group) reveal a more integrated structure of PADM [Figure 6(b–f)]. However, morphological differences are still observed among the crosslinking groups. ADA (OD = 25%) group and DHT + EDC group shows a more rough surface and the pore structure which may be induced by the degradation of collagen fibers within PADM (the local insets of Figure 6(c,f)). Besides, ADA (OD = 45%) group, ADA (OD = 65%) group, and GA group reveal a relatively intact and clear pore structure which are consistent with the degradation data in the above section accordingly. The possible reason why PADM crosslinked by GA and ADA (ADA with 45% and 65% OD value) show slightly different SEM morphology is that the main chain of ADA is much longer than GA or its polymer which could affect the reaction process between the free ε-NH_2 of lysine of collagen within PADM and the aldehyde group within ADA due to stereo-hindrance effect and this phenomenon could also be reflected by the crosslink density results (Table I).[40] However, totally speaking the structure integrity of PADM crosslinked by ADA with 45% and 65% oxidation degree reflected by SEM

Table II. Weight Losses and Residual Weights of PADM Scaffolds before and after Crosslinking during the Heating Process [(a) No Crosslinking Group as the Control, (b) DHT + EDC Group, (c) ADA (OD = 25%) Group, (d) ADA (OD = 45%) Group, (e) ADA (OD = 65%) Group, and (f) GA Group]

Samples	Weight loss (%) at 40-150 °C	Weight loss (%) at 200-800 °C	Residual weight (%)
a	18.2	65.6	16.2
b	15.4	66.5	18.1
c	14.9	64.2	20.9
d	14.8	60.2	25.0
e	14.3	60.6	25.1
f	11.3	63.2	25.5

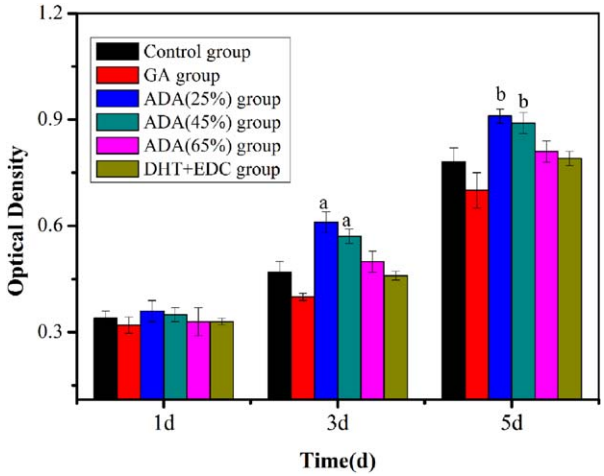

Figure 8. Cell proliferation on PADM scaffolds before and after crosslinking after day 1, 3, and 5. [No crosslinking group as the control, GA group, ADA (OD = 25%) group, ADA (OD = 45%) group, ADA (OD = 65%) group, and DHT + EDC group]. Asterisk (a–b) denotes the difference attained a statistically significant difference compared to the control group at different time period. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

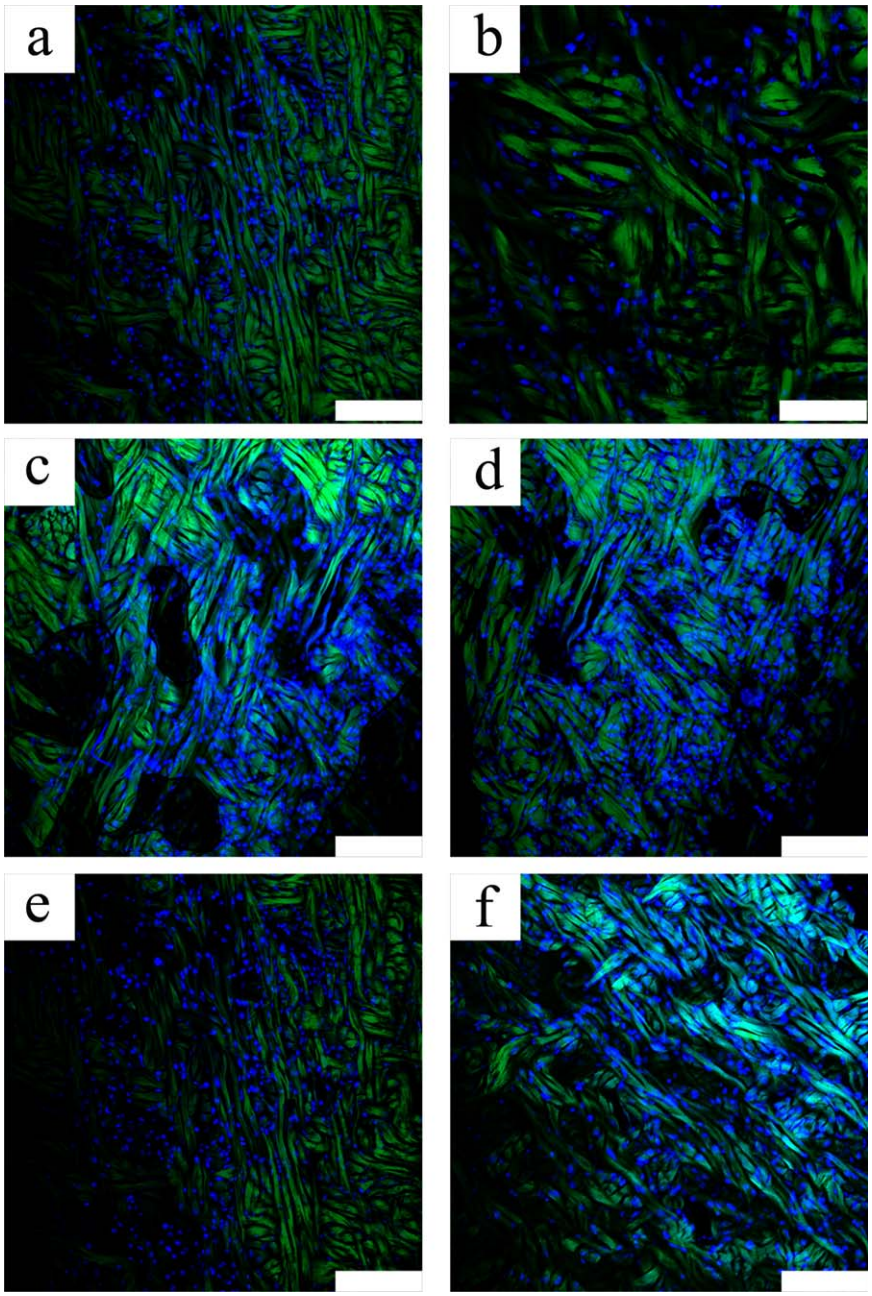

Figure 9. CLSM photographs of L929 fibroblasts cultured for 3 days on PADM scaffolds before and after crosslinking. [(a) No crosslinking group as the control, (b) GA group, (c) ADA (OD = 25%) group, (d) ADA (OD = 45%) group, (e) ADA (OD = 65%) group, and (f) DHT + EDC group]. The scale bar is 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

morphology [Figure 6(d,e)] are well maintained which could be revealed by the integrated pore structure.

Thermal Stability Analysis

Thermogravimetric analysis (TGA) is performed on PADM crosslinked by ADA to determine the thermal stability while GA, EDC, and DHT are used as the control. The curves of weight loss and first derivate related to the rate of weight loss Differential thermal gravity (DTG) thermographs for PADM both crosslinked and noncrosslinked are shown in Figure 7(a,b). The thermal degradation of PADM usually occurs during the testing temperature range (from about 40 °C to 800 °C).[32,38] Typically, two different steps can be observed in the degradation process of PADM scaffolds both before and after crosslinking. During the first step (from about 40 °C to 150 °C), the weight loss of PADM scaffolds ascribes to the breakage of inter- and intra-molecular hydrogen bonds accompanied by gradual loss of water.[26,27] In addition, for PADM before crosslinking, it is mostly related to the disruption of the triple helix of collagen fibers, during the second step (from about 200 °C to 800 °C), the weight loss is assigned to the decomposition of collagen fibers within PADM, whereas for PADM after crosslinking, during the weight losing procedure, it

is also necessary to disrupt the crosslinks between the side chains of collagen molecular within PADM and various crosslinkers, hence we could obtain the information on which kind of crosslinker can give PADM higher thermal stability through the migration of DTG curves. As is shown in Table II and Figure 7(B), most of the weight losses of PADM take place during heating process. Note that, we can see that the thermal denaturation and degradation is hindered by the introduction of ADA from the DTG thermographs, the residual weight for PADM crosslinked by ADA (OD = 45% and 65%) reach up to almost the same value (about 25%) for PADM scaffolds crosslinked by GA, which is much higher than that of control. This phenomenon illustrates that ADA with suitable oxidation could promote the thermal stability that will be benefit for tissue engineering applications.[2,40]

Cytocompatibility Analysis

The cytocompatibility of PADM crosslinked by DHT + EDC, ADA, and GA are evaluated by measuring L929 fibroblasts proliferation and examining cell attachment and distribution in PADM over a period of 5 days. Figure 8 illustrates the cell proliferation results highlighting the significant increase in the growth of L929 fibroblasts among day 1, 3, and 5 for all specimens. However, the proliferation of L929 fibroblasts of GA group are lower than that of control group at day 1, 3, and 5 (5.9% less in day 1, 14.9% less in day 3, and 10.3% less in day 5). Note that ADA (OD = 25%) and ADA (OD = 45%) groups show a relatively higher optical density than that of control group, while ADA (OD = 65%) group reveals a lower optical density than that of ADA (OD = 25%) and ADA (OD = 45%). The reason why ADA with lower oxidation degree (OD) could promote L929 fibroblasts' proliferation, whereas ADA with higher OD instead may be ascribed to two factors. On one hand, alginate dialdehyde was derived from alginate, a natural polysaccharide, which has been demonstrated biocompatible and bio-inductive in many literatures.[7,15,31] On the other hand, aldehyde group is considered to be poor biocompatibility, when the OD of ADA was low, the cytotoxicity of aldehyde group may be masked by the bio-inductive effect of ALG skeleton and PADM, but along with the increasing of OD, the inherent cytotoxicity of aldehyde began to emerge as was shown in ADA (OD = 65%) group.[35,39] Figure 9 presents the L929 fibroblasts' distribution on PADM scaffolds crosslinked by GA, ADA, DHT + EDC, while no crosslinking group as the control. Confocal laser scanning microscopy (CLSM) images show better distribution of L929 fibroblasts on PADM scaffolds crosslinked by ADA (OD = 25%) group, ADA (OD = 45%) group than GA group at day 3, which is in consistence with the MTT assay results (The blue spots represent L929 fibroblasts). Considering the crosslinking effect, resistance ability to enzyme, mechanical property, and cytocompatibility, ADA with the oxidation degree of 45% shows a better integrated performance than other groups. Since this kind of dialdehyde compounds derived from natural biomass combines both the crosslinking feature of glutaraldehyde and the good biocompatibility of natural biomass, ADA with suitable oxidation degree may be developed as the potential biocrosslinker for PADM in future, and this is just what our present work aims to investigate and determine.

CONCLUSIONS

The physicochemical behavior (mechanical property and thermal stability before and after crosslinking, resistance to enzymatic degradation, and 3D hierarchical structure) of a collagenous scaffold (PADM) crosslinked by ADA with different oxidation degree (25%, 45%, and 65%) was investigated *in vitro* in our work. The histological observation and SEM results indicated that PADM crosslinked by ADA with a certain oxidation degree (45% and 65%) possess better stability when exposed to collagenase and the 3D hierarchical structure was also maintained to a large extent. Note that the MTT assay and CLSM results suggested that ADA with certain oxidation degrees (25% and 45%) showed better biocompatibility than GA and DHT + EDC group. In conclusion, the results obtained in this *in vitro* study suggested that ADA could be further explored, as a promising natural derived biological crosslinker for PADM modification, however, more detailed work on the *in vivo* study of PADM modified by ADA are still necessary in our future work.

ACKNOWLEDGMENTS

The financial support of the Fundamental Research Funds for the Central Universities (2662014BQ053, 2662015QC014), Natural Science Foundation of Hubei Province (2015CFB391), National Natural Science Foundation of China (Program NO. 21506070; 51503129), and China Agriculture Research System (CARS-46-23) are gratefully acknowledged.

REFERENCES

1. Hu, Y.; Liu, L.; Dan, W.; Dan, N.; Gu, Z.; Yu, X. *Int. J. Biol. Macromol.* **2013**, *55C*, 211.
2. Zhang, X.; Yang, J.; Li, Y.; Liu, S.; Long, K.; Zhao, Q.; Zhang, Y.; Deng, Z.; Jin, Y. *Tissue Eng. C, Meth.* **2011**, *17*, 423.
3. Zhang, X.; Deng, H.; Wang, Z.; Yang, W.; Guo, Y.; Ma, D.; Yu, C.; Zhang, Y.; Jin, Y. *Biomaterials* **2009**, *30*, 2666.
4. Bian, D.; Chen, M.; Yu, R.; Liu, B.; Zhang, A.; Song, G. *J. Hard Tissue Biol.* **2014**, *23*, 21.
5. Liu, S.; Zhang, H.; Zhang, X.; Lu, W.; Huang, X.; Xie, H.; Zhou, J.; Wang, W.; Zhang, Y.; Liu, Y.; Deng, Z.; Jin, Y. *Tissue Eng. A* **2011**, *17*, 725.
6. Badylak, S. F. *Biomaterials* **2007**, *28*, 3587.
7. Gu, Z.; Xie, H.; Huang, C.; Li, L.; Yu, X. *Int. J. Biol. Macromol.* **2013**, *58*, 121.
8. Takitoh, T.; Bessho, M.; Hirose, M.; Ohgushi, H.; Mori, H.; Hara, M. *J. Biosci. Bioeng.* **2015**, *119*, 217.
9. He, L.; Theato, P. *Eur. Polym. J.* **2013**, *49*, 2986.
10. Aston, R.; Wimalaratne, M.; Brock, A.; Lawrie, G.; Grondahl, L. *Biomacromolecules* **2015**, *16*, 1807.
11. Zhang, M.; Ding, C.; Huang, L.; Chen, L.; Yang, H. *Cellulose* **2014**, *21*, 3311.
12. Jeong, S. I.; Krebs, M. D.; Bonino, C. A.; Khan, S. A.; Alsberg, E. *Macromol. Biosci.* **2010**, *10*, 934.

13. Balakrishnan, B.; Jayakrishnan, A. *Biomaterials* **2005**, *26*, 3941.
14. Campi, G.; Fratini, M.; Bukreeva, I.; Ciasca, G.; Burghammer, M.; Brun, F.; Tromba, G.; Mastrogiacomo, M.; Cedola, A. *Acta Biomater.* **2015**, *23*, 309.
15. Safandowska, M.; Pietrucha, K. *Int. J. Biol. Macromol.* **2013**, *53*, 32.
16. Sarker, B.; Papageorgiou, D. G.; Silva, R.; Zehnder, T.; Gul-E-Noor, F.; Bertmer, M.; Kaschta, J.; Chrissafis, K.; Detsch, R.; Boccaccini, A. R. *J. Mater. Chem. B* **2014**, *2*, 1470.
17. Xu, Y.; Huang, C.; Li, L.; Yu, X.; Wang, X.; Peng, H.; Gu, Z.; Wang, Y. *Carbohydr. Polym.* **2013**, *95*, 148.
18. Xu, Z.; Shi, L.; Yang, M.; Zhang, H.; Zhu, L. *J. Mater. Chem. B* **2015**, *3*, 3634.
19. He, L.; Cai, S.; Wu, B.; Mu, C.; Zhang, G.; Lin, W. *J. Inorg. Biochem.* **2012**, *117*, 124.
20. Hu, Y.; Liu, L.; Dan, W.; Dan, N.; Gu, Z. *J. Appl. Polym. Sci.* **2013**, *130*, 2245.
21. Yahyouche, A.; Zhidao, X.; Czernuszka, J. T.; Clover, A. J. P. *Acta Biomater.* **2011**, *7*, 278.
22. O'Brien, F. J.; Haugh, M. G.; Murphy, C. M.; McKiernan, R. C.; Altenbuchner, C. *Tissue Eng. A* **2011**, *17*, 1201.
23. Powell, H. M.; Drexler, J. W. *Tissue Eng. C Meth.* **2011**, *17*, 9.
24. Hu, Y.; Liu, L.; Dan, W. *J. Soc. Leather Technol. Chem.* **2013**, *97*, 200.
25. McPherson, J.; Sawamura, S.; Armstrong, R. *J. Biomed. Mater. Res.* **1986**, *20*, 93.
26. Fathima, N. N.; Madhan, B.; Rao, J. R.; Nair, B. U.; Ramasami, T. *Int. J. Biol. Macromol.* **2004**, *34*, 241.
27. Cheung, D. T.; Perelman, N.; Ko, E. C.; Nimni, M. *Connect. Tissue Res.* **1985**, *13*, 109.
28. Roessner, E. D.; Thier, S.; Hohenberger, P.; Schwarz, M.; Pott, P.; Dinter, D.; Smith, M. *J. Biomater. Appl.* **2011**, *25*, 413.
29. Gu, Z.; Zhang, X.; Yu, X.; Li, L.; Xu, Y.; Chen, Y. *Mater. Sci. Eng. C Mater. Biol. Appl.* **2011**, *31*, 1593.
30. Ahmadi, A.; Thorn, S. L.; Alarcon, E. I.; Kordos, M.; Padavan, D. T.; Hadizad, T.; Cron, G. O.; Beanlands, R. S.; Dasilva, J. N.; Ruel, M. *Biomaterials* **2015**, *49*, 18.
31. Xu, Y.; Li, L.; Wang, H.; Yu, X.; Gu, Z.; Huang, C.; Peng, H. *Carbohydr. Polym.* **2013**, *92*, 448.
32. Bottino, M. C.; Thomas, V.; Jose, M. V.; Dean, D. R.; Janowski, G. M. *J. Biomed. Mater. Res. B: Appl. Biomater.* **2010**, *95B*, 276.
33. Wang, H.; Blitterswijk, C. A. V. *Biomaterials* **2010**, *31*, 4322.
34. Ding, C.; Zhang, M.; Li, G. *Carbohydr. Polym.* **2015**, *119*, 194.
35. Vidal, C. M.; Leme, A. A.; Aguiar, T. R.; Phansalkar, R.; Nam, J. W.; Bisson, J.; Mcalpine, J. B.; Chen, S. N.; Pauli, G. F. *Langmuir* **2014**, *30*, 14887.
36. Gouveia, R. M.; Jones, R. R.; Hamley, L. W.; Connon, C. *J. Biomater. Sci.* **2014**, *2*, 1222.
37. Zhao, H.; Wang, G.; Hu, S.; Cui, J.; Ren, N.; Liu, D.; Liu, H.; Cao, C.; Wang, J.; Wang, Z. *Tissue Eng. A* **2011**, *17*, 765.
38. Jin, Y.; Deng, Z. H.; Wu, J. J.; Qiu, J. H.; Wang, J. L.; Tian, Y. S.; Li, Y. *Tissue Eng. A* **2009**, *15*, 3729.
39. Ma, J.; Sahoo, S.; Baker, A. R.; Derwin, K. A. *J. Biomed. Mater. Res. B: Appl. Biomater.* **2015**, *103*, 335.
40. Fratzl, P. Collagen: Structure and Mechanics; Springer Science + Business Media: New York, **2008**.