Collagen and Leather

RESEARCH

Open Access

Multi-functional epoxides cross-linked collagen sponges for tissue engineering scaffolds

Yuanzhi Zhang[1], Changkai Yang[1], Min Gu[1], Xiaoxia Zhang[2], Xueqing Zhang[3] and Guoying Li[1,2*]

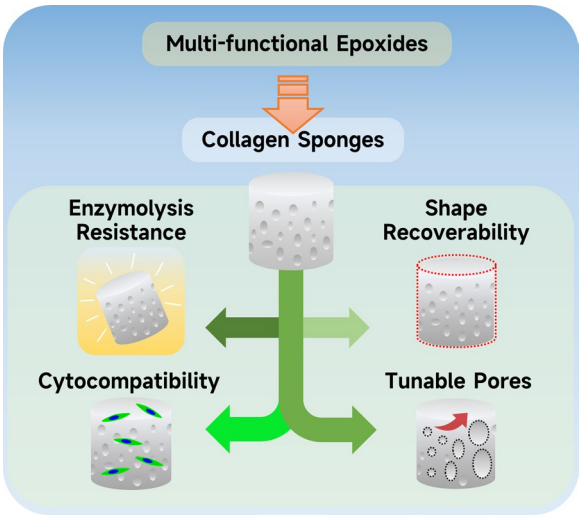
Abstract

With the efficient cross-linking abilities and the flexible regulation abilities to the performances of cross-linked products, the multi-functional aliphatic epoxides were once widely used to cross-link the collagen-based materials in the last century. In present work, the multi-functional epoxides were used to construct and cross-link collagen sponges for tissue engineering scaffolds, which was hoped to board the theoretical system of epoxides and explore their potentials for modern applications. The bi- to tetra-functional epoxides were used to cross-link collagen solutions and establish the gel-like precursors, then using freeze-drying to form the final sponges. The SEM observed that the sponges had shown regular porous structures with a wide range of pore sizes from 160 to 440 μm. The sponges had presented the resistance to enzymatic degradation, shape-remaining ability, and reversible compressibility in aqueous environments, which all could be regulated through the functionalities of epoxides. The regulation abilities of multi-functional epoxides on the performances of sponges had been mainly achieved through the cross-linking degrees that the higher functionality of epoxides would bring higher cross-linking degree. Such higher cross-linking degrees could enhance the elastic behaviors of gel-like precursors, and improve the compressive strengths and thermal stabilities of sponges. Nevertheless, the multi-functional epoxides had barely affected the safety of collagen sponges at the cellular level according to the results of CCK8 assay and the SEM and CLSM images of L929 fibroblasts cultured on the cross-sections of sponges.

Keywords Multi-functional epoxides, Collagen sponges, Tunable porous structures, Reversible compressibility

*Correspondence:
Guoying Li
liguoyings@163.com
Full list of author information is available at the end of the article

Graphical abstract



1 Introduction

The multi-functional epoxides, which are known for the ability to establish strong cross-linking networks, have been widely used in the fields of adhesion, coating, textile, and papermaking for decades [1, 2]. Collagen is the major component of extracellular matrix (ECM) [3, 4], which has accepted bioactivity and an abundance of functional groups including amino, carboxy, and hydroxy groups that makes collagen the common biomaterial for tissue engineering [5, 6]. Benefited from the good reaction ability between the epoxy groups and amino groups of collagens, the epoxides have been used to reinforce the collagen-based materials, which could be traced back to 1950s where the epoxides were deployed as leather tanners [7, 8]. When it came to the 1990s, the aliphatic epoxides were applied on natural animal tissues such as tendons, pericardium, arterial valves, etc. to develop collagen-based xenografts [9–11].

As the collagen cross-linkers with good potentials, the multi-functional epoxides could enhance the mechanical properties, thermal stabilities, and enzymatic resistance of cross-linked products with acceptable biocompatibility including non-cytotoxicity, non-mutagenicity and resistance to calcification [12–14]. Besides, different from the traditional bi-functional cross-linkers such as GA or NHS, the epoxides containing multiple active epoxy groups are able to construct three-dimensional networks rather than the two-dimensional bridging systems [15]. Moreover, the multi-functional epoxides could regulate the performance of cross-linked products more flexibly through manipulating the cross-linking degrees according to their functionalities, while the regular bi-functional cross-linkers usually depended on the dosage. For such capabilities to construct three-dimensional collagen cross-linking networks that were efficient, simple, and customizable, the epoxides were now used to prepare and reinforce the tissue engineering scaffolds. Bock et al. used 1,4-butanediol ether to cross-link the hydroxyapatite/collagen scaffolds that dip-coated by aqueous ferrofluids containing iron oxide nanoparticles to prepare the magnetic biomimetic scaffolds who presented the ability to support adhesion and proliferation of human bone marrow stem cells in vitro [16]. Zheng et al. used the epoxidized natural polysaccharide to prepare the highly stable collagen scaffolds based on porcine acellular dermal matrix, the thermal stability and mechanical properties of scaffolds were remarkably improved without adverse effects on the biocompatibility of the materials [17]. Minardi et al. fabricated a magnesium-doped hydroxyapatite/collagen scaffold that was wet cross-linked by 1,4-butanediol digylcidyl ether, the scaffolds had successfully induced osteogenic differentiation in vitro and promoted the bone regeneration in clinically-relevant scenarios in vivo [18].

Other than the reinforcements on collagen-based biomaterials, the multi-functional epoxides also had extra advantages that the grafted epoxy groups on collagen molecules would be rarely hydrolyzed and remain their good reaction activity [19]. On the one hand, the active epoxy groups could help collagen to connect and composite other biomaterials containing available groups

to fabricate heterogeneous composites [20]. Kim et al. developed hybrid scaffolds composed of hyaluronic acid and collagen, where the ethylene glycol diglycidyl ether could react to both of the amino groups of collagens and the hydroxy groups of hyaluronic acid. The results showed that the scaffolds had three-dimensional structures with interconnected pores and effectively promoted the cartilage regeneration [21]. On the other hand, the grafted epoxy groups could be able to help the cell adhesion. Trimbach et al. prepared an epoxide-functionalized surfaces to provide new routes for cost-effective coatings, and their results demonstrated that the epoxide groups on the glass surfaces even outperformed ECM protein gel in cell adhesion [22]. In another recent report, the epoxides cross-linked collagen-based bio-interfaces had also presented better ability to enhance the strength of cell adhesion, who also mentioned that the enhancement might originate from the bonding between epoxy groups and proteins on the surfaces of cells [23].

The epoxides had proved their potentials and capabilities during the long-time applications; however, there were still unexplored areas that mattered to the practices. From a fundamental perspective, the theoretical researches were basically carried out in the last century and based on the nature tissues. The tissues from different parts of different animals had their own natural properties, which led to different outcomes from different researches. For instance, the shrinkage temperature of cross-linked porcine tendon would be about 8 °C higher than bovine pericardium even though the reaction conditions and epoxides used were the same [24, 25]. Another problem was that the applications nowadays focused more on the artificial advanced constructs of collagen such as hydrogels, sponges, and films, which were always started from extracted dispersed collagen molecules. Whereas, the investigations based on animal tissues, where the collagen had already existed in the original high structures, might not be sufficient enough to predict and guide the development and designing of modern collagen-based materials. In addition, the modern investigations tended to directly employ epoxides to reinforce the collagen-based biomaterials just as many other regular cross-linkers did, which left the regulation potentials of multi-functional epoxides not exploited and applied sufficiently.

In this work, 4 kinds of aliphatic epoxides (as shown in Table 1) were selected to cross-link bovine skin Type I collagen to fabricate the collagen sponges for tissue engineering. Combined with the discussions around both the sponges and corresponding gel-like precursors, series properties of epoxides cross-linked collagen, such as the morphologies, cross-linking degrees, mechanical properties, and cytocompatibilities, which mattered to the designs and applications, had been investigated.

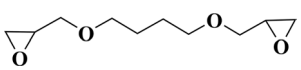
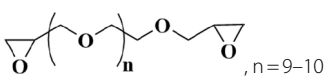
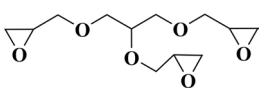
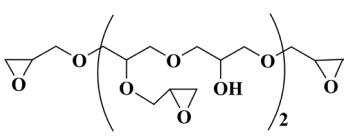
2 Materials and methods

2.1 Materials

Bovine skin Type I collagen was extracted from the fresh calf skins based on our previous work [26]. In brief, the fresh skin was first defatted, neutralized and cut, then the small pieces of skin were soaked in 0.5 M acetic acid containing 3% pepsin (1:3000, based on dry weight). After stirring for 48 h, 0.7 M NaCl solution was added to salt out natural Type I collagen at 4 °C. The precipitate was separated by centrifuge and redissolved using 0.5 M acetic acid. The collagen solution was purified by dialyzing at 4 °C and lyophilized for storing at last.

The 1,4-butanediol diglycidyl ether (BDDGE) was provided by Weng Jiang Reagent Co. Ltd. (Shaoguan, China; purity > 95%), the poly (ethylene glycol) diglycidyl ether was provided by Sigma-Aldrich, the glycerol polyglycidyl

Table 1 The structures of epoxides

Epoxides	Amount of epoxy groups	Molecular weight	Structural formulas
1,4-butanediol diglycidyl ether	2	174	
Poly (ethylene glycol) diglycidyl ether	2	~ 500	
Glycerol polyglycidyl ether	3	232	
Polyglycerol polyglycidyl ether	4	538	

ether and polyglycerol polyglycidyl ether were donated by Ao Ke Advanced Materials Co. Ltd. (Shanghai, China). The corresponding structures were listed in Table 1.

2.2 Preparation of the epoxides cross-linked collagen sponges

Lyophilized collagen was dissolved in 0.036 M sodium carbonate/0.064 M sodium hydrogen carbonate ($NaHCO_3/Na_2CO_3$) buffer solution (pH = 10.68). The initial concentration of collagen solution was 1.25-wt% and the epoxides were prepared as the solution of 20-wt%, then the epoxide solutions were added dropwise to the collagen solutions until the final concentrations of collagen and epoxides were 1-wt% and 4-wt%, respectively, in mixtures. The samples were first stirred for 30 min at 4 °C and then transferred to 24-well plates and left them still for 24 h to form the gel-like precursors at room temperature, followed by immersed in ultrapure water for purification. The water was changed each hour until the conductivity of water was lower than 20 μS/cm after immersing. The purified gel-like precursors were first fully frozen for 24 h, then lyophilized under −30 °C in 0.37 mpa for 48 h to obtain the sponges. According to the amounts of epoxy groups that the epoxides containing, the sponges were coded as Col-Ep2, Col-Ep3, Col-Ep4. The sponges that were cross-linked by poly (ethylene glycol) diglycidyl ether who was the bi-functional epoxide with the longest backbone were coded as Col-Ep2L.

2.3 Microstructure of epoxides cross-linked collagen sponges

2.3.1 Morphological analysis

The porous structures were important characteristics of sponges, which were analyzed by a Hitachi SU3500 scanning electron microscope (SEM, Hitachi, Tokyo, Japan). After treated by liquid nitrogen, the sponges were separated vertically to expose the interior structure. Then the samples were coated with an ultrathin layer of gold–palladium using an ion sputter. The observation was achieved at an accelerating voltage of 15 kV. Based on the SEM images, the pore sizes were measured using software ImageJ (National Institutes of Health, USA), and each sample was measured from 3 different positions.

2.3.2 Swelling ratio

The swelling ratios of the gel-like precursors were related to the final porous structures of sponges and helped to observe the property differences among the cross-linking networks established by different functional epoxides. The initial weights (W_i) of gel-like precursors were measured immediately when the precursors formed, then the gel-like precursors were immersed in deionized water at 37 °C. At predetermined intervals, the gel-like precursors were gently wiped with filter paper and weighted. When the weights of all samples became constant, the final weights (W_f) were measured. The swelling ratio (%) was calculated using Eq. (1):

$$\text{Swelling ratio (\%)} = \frac{W_f - W_i}{W_i} \times 100 \quad (1)$$

2.4 Evaluations of the durability and cross-linking degree

2.4.1 Thermal stability

The thermal stability was measured by differential scanning calorimeter (Netzsch 200PC, Selb, Germany). 5 mg of each sponge was sealed in an identical pan and the thermal scanning was performed between 25 and 100 °C at a heating rate of 5 °C/min under a nitrogen atmosphere with an empty pan as reference.

2.4.2 Enzymatic stability

The enzymatic stability was analyzed using Type I collagenase from *Clostridium histolyticum* (≥125 U/mg, C0130, Sigma-Aldrich, Munich, USA). The sponges were firstly weighted (W_0), then immersed in 10 mL of collagenase solutions (5 U/mL) for 1, 2, 4, 8, and 12 h under 37 °C. At each time point, the residual sponges were taken out from the collagenase solutions and washed using ice ultrapure water immediately to terminate the enzymatic degradation. The residual sponges were weighted again (W_1) after freeze-drying again. The enzymatic stability could be expressed as the enzymatic degradation degrees by Eq. (2):

$$\text{The enzymatic degradation degree (\%)} = 1 - \frac{W_1}{W_0} \times 100 \quad (2)$$

2.4.3 Determination of the reacted amino group content

The trinitrobenzensulfonic acid (TNBS) assay was used to measure the reacted amino group content of sponges. 0.15 g of sponge was added to 1.2 mL borax buffer (pH = 10.00) and 1.2 mL freshly prepared 0.1% (v/v) TNBS and reacted in the dark at 50 °C for 60 min. Then 4 mL 0.1N HCl was added and kept at room temperature for 30 min. The absorbance of the final solution was recorded at 340 nm using a UV spectrophotometer (PerkinElmer Lambda 25, Waltham, MA). All the samples were measured in triplicate. The reacted amino group content of the cross-linked collagen was calculated according to Eq. (3):

$$\text{The reacted amino group content (\%)} = 1 - \frac{\text{absorbance}_{cc}}{\text{absorbance}_{uc}} \times 100 \quad (3)$$

where the subscripts *cc* and *uc* stand for the cross-linked and uncross-linked collagen, respectively.

2.5 Evaluations of the mechanical properties

2.5.1 Unconfined compressive test

The unconfined compressive tests were performed by Electronic Universal Testing Machine UTM2102 (Shenzhen SUNS Technology Stock CU., LTD, Shenzhen, China) according to GB/T 1041-92. The load and displacement were set to 50 N and 8 mm, respectively, and compressing speed was 5 mm/min. The gel-like precursors were measured only in air, while the sponges were measured in air and ultrapure water to observe the shape recovery properties as references [27].

2.5.2 Dynamic rheological measurements

To better understand the effects of epoxides with different functionalities to the cross-linking network, the dynamic rheological measurements of gel-like precursors were performed on a Rheometer System Gemini 200 (Molvern Instruments, Malvern, UK). The gap between parallel plate geometry (40 mm diameter) was set at 5 mm in all tests. The frequency of dynamic frequency scanning was set from 0.01 to 10 Hz at 25 °C at a constant strain of 5%. The temperature was controlled by a Peltier temperature controller with an accuracy of ± 0.1 °C. Dynamic stress sweeps were conducted prior to the frequency sweeps to ensure operation within the linear viscoelastic region. Storage modulus (G′), loss modulus (G″) were recorded as a function of the frequency.

2.6 In vitro cytocompatibility evaluations

The L929 fibroblasts were used to evaluate the in vitro cytocompatibility based on the guidance of ISO10993, and the samples were irradiation sterilized by gamma ray in advance. The sponges were extracted as the ratio of 0.2 g/mL at 37 °C for 72 h as the relevant requirements. The cells were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium containing 10%(v/v) bovine calf serum (BCS) and 1% penicillin/streptomycin at 37 °C in 95% air/5% CO_2. The L929 cells were used at passages 3–8 and the culture medium was changed every 2 days during this process.

The cytotoxicity of extracts of radiation sterilized samples was performed by CCK-8 colorimetry. The cell suspensions were diluted by culture medium and added 100 μL to 10^4 cells/well in a 96-well plate and incubated for 4 h at 37 °C in an atmosphere of 95% air/5%CO_2, then cultured with extracted liquids for 1, 3, 5 days, respectively. At each time point, 10 μL of CCK-8 was added to each well and the optical densities (OD) were recorded using a Multiskan FC microplate reader (Mindray, MR-96A, China) at 450 nm. The values were expressed as mean ± standard deviation.

The cell morphologies were observed by inverted fluorescence microscope (ECLIPSE, Ti2-U, Nikon, Japan) based on L929 fibroblasts seeded in the cross-sections of sponges. The radiation sterilized samples were formed in 6-wells plates in advance and the L929 fibroblasts with density of 5×10^4/dishes were seeded in each well. After 3 days of culturing, the mixed solutions containing 10 μg/mL rhodamine-phalloidin were added and incubated for 20 min. Besides, the cells cultured after 5 days were observed by a Hitachi SU3500 scanning electron microscope (SEM, Hitachi, Tokyo, Japan). During the culturing process, the temperature of the environment remained at 37 °C and the atmosphere was kept as 95% air/5%CO_2, and the medium was changed each 48 h.

2.7 Statistical analysis

All measurements were performed in triplicate to obtain mean values ± standard deviations. One-way ANOVA was performed in SPSS 20 software to analyze the variables, and $p < 0.05$ was considered statically significant.

3 Results and discussion

3.1 Microstructural analysis

Different from the natural tissues that had certain anatomical structures and functions, the actual structures and performances of artificially produced bio-materials used for tissue engineering such as the sponges, hydrogels, or films, depended more on the preparation technologies. During the preparation process of collagen sponges, the raw materials, freeze-drying conditions, cross-linkers, would all affect the final structures and performances [28]. In present work, the collagen was all extracted from bovine skins, and the freezing and drying process of gel-like precursors were all the same. Therefore, the differences among the morphologies of sponges presented in Fig. 1 would be mainly affected by the epoxides used.

The regular porous structures of epoxide cross-linked sponges had similar general structures as the reports of collagen-based biomimetic scaffolds used for tissue engineering [29, 30], while the untreated collagen exhibited relatively unordered and mussy structures. It can be seen that from Col-Ep2 to Col-Ep4, who were cross-linked by the epoxides with gradually increasing functionalities, the porous structures of sponges were getting tight and the pore sizes appeared to be shrunk. By contrast, the Col-Ep2L that cross-linked by a bi-functional epoxide

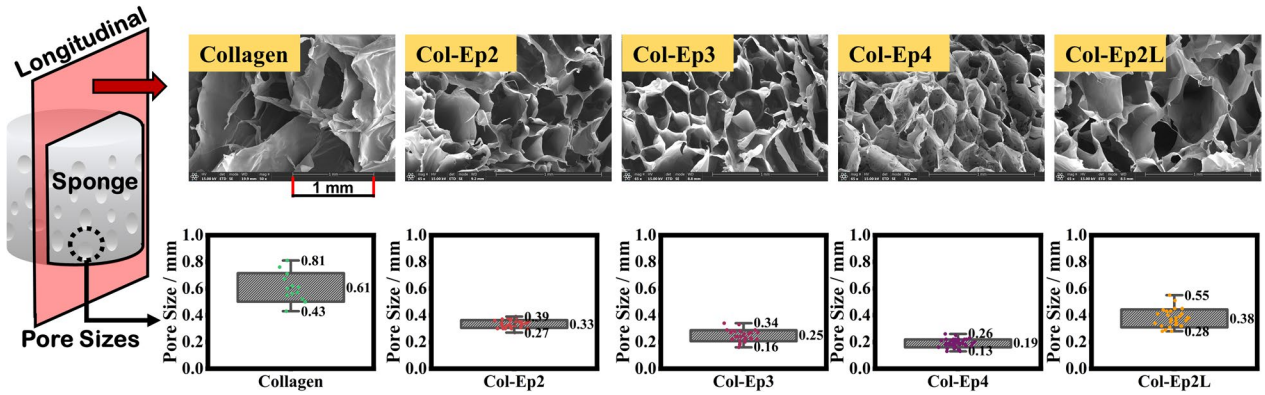


Fig. 1 The microstructures of collagen sponges cross-linked by multi-functional epoxides, which were observed using SEM, scale bar: 1 mm, and the corresponding pore sizes were counted based on SEM images

with the longest backbone, had the looser structures compared with other cross-linked sponges. To quantitatively present the differences among the pores of different sponges, the pores sizes were also counted based on SEM images and shown in Fig. 1. It was obvious that with higher functionalities of epoxides used, the average pore sizes of sponges had reduced from the 0.33 mm of Col-Ep2 to the 0.25 mm of Col-Ep3 and further to 0.19 mm of Col-Ep4. Meanwhile, the distributions of pore sizes also tended to be concentrated shown as flat boxes in corresponding box plots from Col-Ep2 to Col-Ep4, which indicated much tighter and more regular structures. By comparison, the Col-Ep2L had a much wider distribution of pore sizes that echoed its looser structure.

The different structures of sponges were proofs of the regulatory ability of multi-functional epoxides. Under the similar chemical cross-linking mechanism and reaction conditions, the epoxides could achieve wider and finer controls on the structures of collagen sponges. The similar controls on cross-linking might be more complex for the single-form chemical cross-linkers such as glutaraldehyde, genipin, and EDC/NHS, etc. that relied more on the dosages. As for practical applications, the pore sizes of multi-functional epoxides cross-linked sponges had covered a wide range from 190 μm (Col-Ep4) to 380 μm (Col-Ep2L), which could meet more extensive demands of biomedical applications including vascularization, bone regeneration, cartilage regeneration, and dermal substitutes [31–33]. From the theoretical perspective, the morphologies of cross-linked sponges had revealed the regulatory ability of multi-functional epoxides on the structures of collagen-based artificial biomaterials. In the past, whether the structures of epoxides were, the structures of cross-linked animal tissues would always be tighter due to the effects of chemical cross-linking or physical filling [24]. The results about functionalities still suitable for sponges, considering that the higher functionalities of epoxides could form the tighter cross-linking networks according to the trends of Col-Ep2, Col-Ep3, and Col-Ep4. The long backbones of epoxides were believed to be able to fill the inner spaces in animal tissues and enhance the stabilities and mechanical properties. However, it seemed that the long backbones of epoxides would lose the structures of sponges rather than filling the interior spaces, especially considering the different morphologies of Col-Ep2L and Col-Ep2.

The media transforming process from the water of gel-like precursors to the air of sponges was achieved through freeze-drying process. The freezing of water would form ice crystals inside precursors and affect the porous structures of sponges, so that the water capacities of gel-like precursors also mattered to the final sponges [34]. The swelling behaviors of gel-like precursors were shown in Fig. 2. It can be seen that basically all the gel-like precursors had finished the swelling process within immersing for 6 h. The Col-Ep4 who had the smallest pore sizes also exhibited lowest swelling ratio, while the Col-Ep2L, whose pores were the largest ones, had swelled much more significantly.

It can be seen that the swelling ratios of gel-like precursors were corresponding to the final pore sizes of sponges, as shown in Fig. 2b. The correspondence indicated that the foundation of final structural properties of sponges was long laid since the formation of their precursors. Past investigation proved that the higher functionalities of epoxides could bring higher cross-linking degrees, which might further be able to establish stronger and tighter cross-linking networks of gel-like precursors that could resist the swelling. With lower swelling degree, less water would be contained in the precursors and less ice crystals would form so that the final porous structures of sponges would tighter, under other consistent

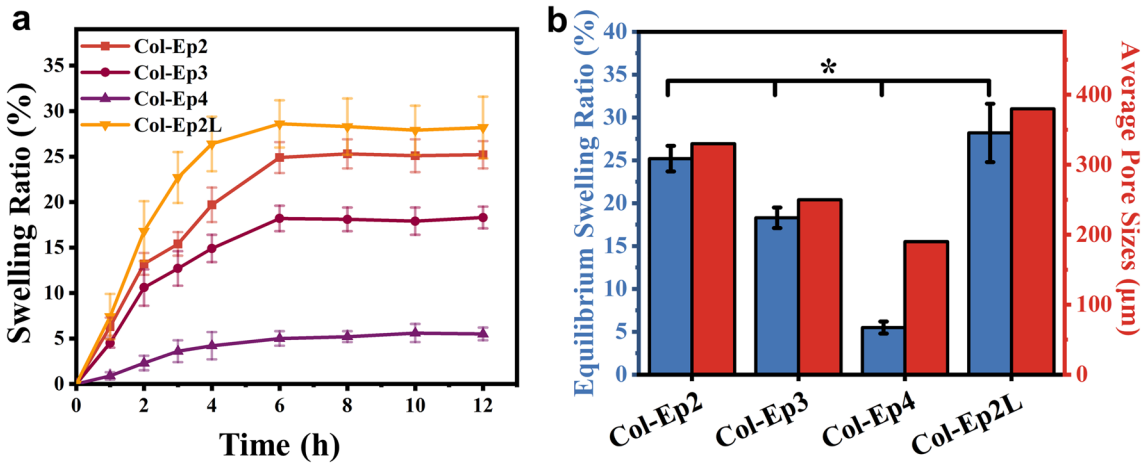


Fig. 2 **a** The swelling behaviors of the gel-like precursors; **b** The equilibrium swelling ratios and corresponding average pore sizes of the gel-like precursors

conditions like the freezing or drying process. As for Col-Ep2L, on the one hand, it was speculated that the long backbones had hampered the cross-linking so Col-Ep2L had the lowest cross-linking degrees and the loosest cross-linking network that would swell significantly. On the other hand, the long backbone might also lead to the longest cross-linking bonds, so the cross-linked collagen molecules could slip to each other in a wider range that led to more significant swelling of gel-like precursors. Still, the losing effects caused by long backbones could be made up by higher functionality. Col-Ep4 had presented the weakest swelling and tightest porous structures, who was cross-linked by an epoxide that had the similar length of backbone to the one used for Col-Ep2L but tetra-functional.

3.2 Mechanical property and recoverability

The mechanical performance was essential to the application of collagen sponges for tissue engineering scaffolds and also helped to understand the effects of multi-functional epoxides on the artificial collagen-based biomaterials. In the constant range of strain up to 65%, it can be seen in Fig. 3a that the compressive stress of sponges had the same trend with the functionalities of epoxides used for cross-linking. The Col-Ep4 that cross-linked by tetra-functional epoxide had the highest stress while Col-Ep2 that cross-linked by bi-functional one behaved relatively weaker. The higher functionalities could bring higher cross-linking degrees, tight structures, and further enhance the mechanical strength of cross-linked collagen-based materials.

Similar results had also been found in the researches based on animal tissues which pointed that the denser structures might be beneficial to the dissipation of external pressures and energy, so the cross-linked tissues were given better mechanical strength [35]. It can be seen that the compress stresses of Col-Ep2, Col-Ep3, and Col-Ep4 were gradually enhanced, and the corresponding epoxides also had the gradually increased functionalities. Besides, the reversible compressibility of collagen sponges was presented in Fig. 3b, and it can be seen that the Col-Ep2L could barely fully recovered while the rest ones had basically been back to original heights. The results indicated that the higher functionalities of epoxides may also facilitate the shape recoverability of sponges. It was also reported that the long backbones of epoxides could filled up the spaces among collagen molecules and hinder their relative sliding, so that even the monofunctional epoxides, which could not form cross-linking, were able to enhance the mechanical strength of cross-linked tissues [36]. However, Col-Ep2 and Col-Ep2L, who were cross-linked by bi-functional epoxides, had the rather similar compress stresses and modules, indicated that the backbone lengths of epoxides may affect the mechanical properties of sponges much slighter. Compared the Col-Ep2L and Col-Ep4, which were both cross-linked by epoxides with long backbones, it was obvious that the functionalities had more significant effects on the mechanical strengths of sponges.

Nowadays, the multi-functional epoxides had usually played as common cross-linkers to the artificial collagen-based biomaterials and rarely shown their capabilities or explored the potentials like they had done to animal tissues back last century. Therefore, it was rather enlightening to find that the multi-functional epoxides could also endow the collagen sponge the shape recoverability under water. The photos in Fig. 4 had directly shown the reversible compressibility of sponges, which

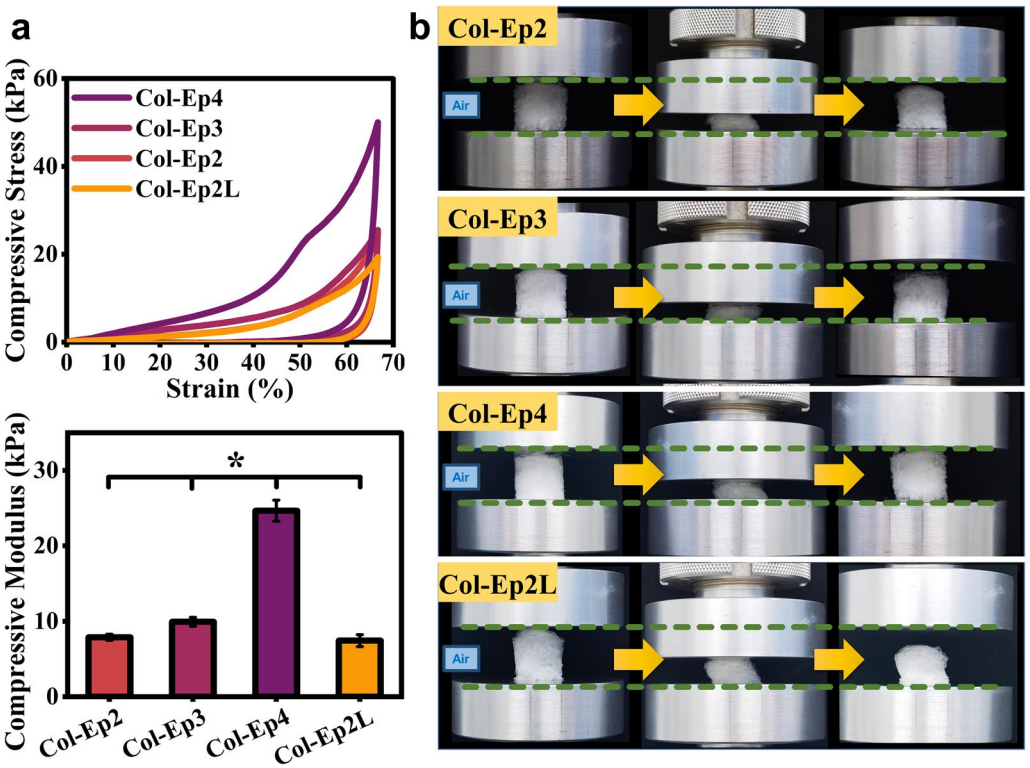


Fig. 3 **a** The compressive stress–strain curves of collagen-based sponges and the corresponding compressive modulus evaluated by the range of stress–strain curves from 0 to 40% with $R^2 > 0.99$; **b** The reversible compressibility presented as the recovery process during compressive test

can be seen that the profiles and shapes of sponges were barely changed by compression. Furthermore, the underwater compression curves of sponges were also provided beside the photos, which were finished by a tensile machine. The corresponding shape recovered curves demonstrated that the compressive strengths of sponges would have a few reductions due to the inevitable structural loss during repeated compression. It can be seen that the higher cross-linking degrees could help the sponges resist the compression that the compressive strength of Col-Ep4 had barely changed while the compressive strength of Col-Ep2L was reduced more distinct.

The multiple level mechanical performance presented above had demonstrated the good potentials of multifunctional epoxides to reinforce and regulate the collagen sponges, and shape-recoverability was an added bonus that allowed the sponges to fit the practical demands of tissue engineering. In addition, the mechanical strength of pure lyophilized collagen was considered to be weak [37], which always required the cross-linking to enhance. The compressive modulus of epoxides established sponges could reach about 10.3 kPa of Col-Ep4 at the strain of 40%, which was higher than the approximate 3.5 kPa of the EDC/NHS cross-linked collagen scaffolds reported by Salim A et al. for tissue engineering [38]. Besides, through integrating other biomaterials like elastin [39], glycosaminoglycan, and hydroxyapatite, etc. [40], it can be expected that the epoxides cross-linked collagen sponges could achieve stronger mechanical strength and extend potentials in more fields.

The mechanical properties of gel-like precursors also had the direct correspondence to the final sponges, which were shown in Fig. 5. Different from the sponges, the gel-like precursors would directly end up with cracking under compression as shown in Fig. 5a and b, yet it also gave another view to observe the effect of epoxides on the mechanical performance. It could be deduced that the precursors of Col-Ep2 and Col-Ep3 were more brittle that cracked around 30% of strain, while the ones of Col-Ep4 and Col-Ep2L were ductile and cracking when framed nearly 40%. Although the compressive modulus had a similar trend with Fig. 3 that Col-Ep4 was the strongest and Col-Ep2L was the weakest, they both exhibited good ductility that might be associated with the long backbones of the epoxides they used, which allowed the collagen molecules to remain connected in larger deformation range.

Meanwhile, the rheological evaluations based on dynamic viscoelasticity of gel-like precursors in Fig. 5c

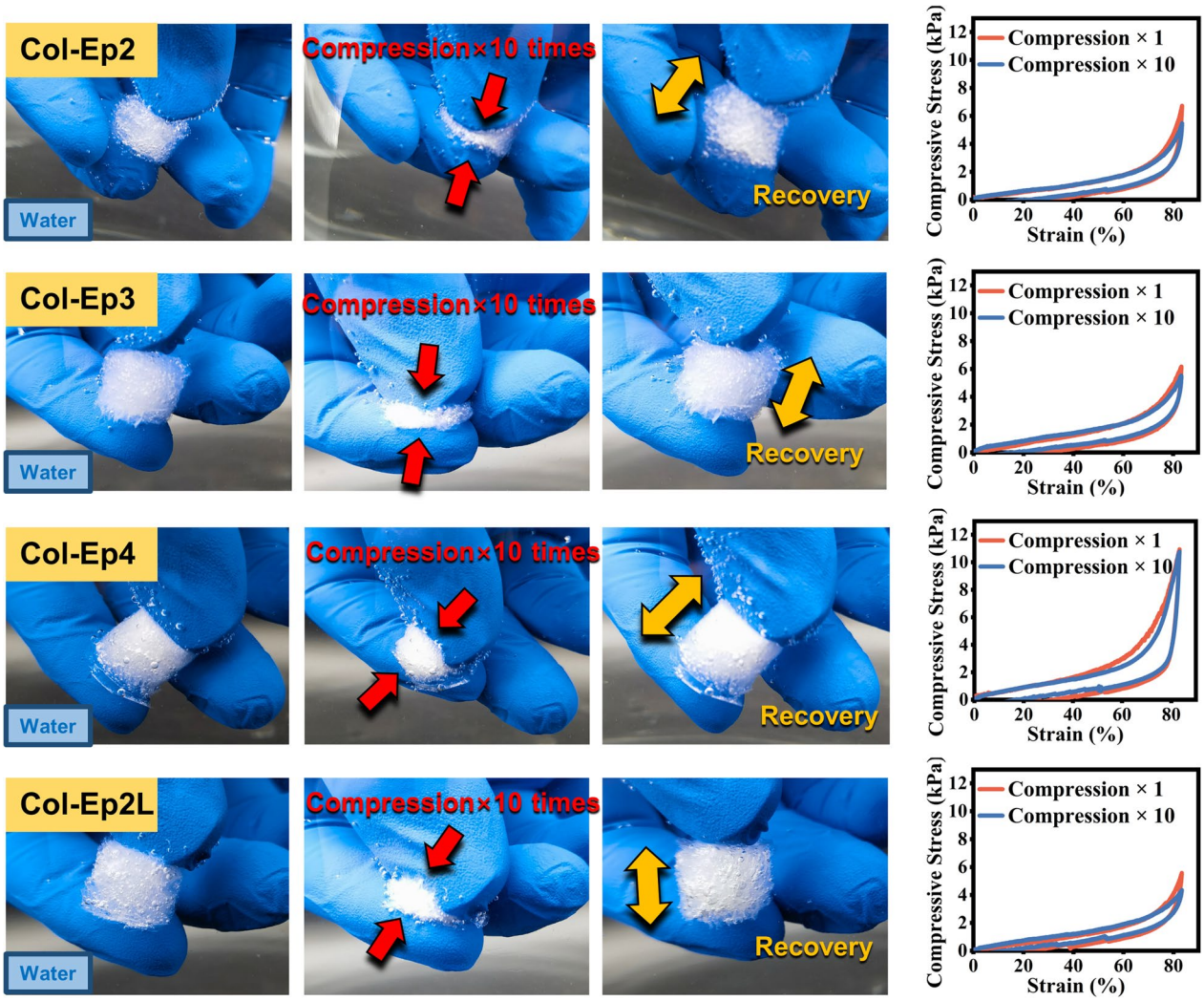


Fig. 4 The reversible compressibility and the corresponding compression curves of sponges in water

had presented that the gel-like precursors from Col-Ep2, to Col-Ep3, till Col-Ep4 were gradually exhibiting higher G′, G″, and tan δ, which indicated the increasing gel-like behaviors. The changes on rheological behaviors had provided another view to explain the rougher and tougher mechanical properties of sponges was that the increasing elasticity modulus. It indicated that the sponges cross-linked by higher functional epoxides would have stronger ability to absorb and store outside energies and remain intact structures to avoid releasing energy by breaking interior structures.

3.3 Durability and cross-linking degrees

The multi-functional epoxides were believed to be the ideal alternative of glutaraldehyde back last century, one of the reasons was that the epoxides could efficiently enhance the thermal and enzymatic stabilities of target tissues. Such enhancements mattered to tissue engineering because the sufficient durability meant the long enough service life under the harsh physiological conditions.

Figure 6 presented the residual weight percentages and corresponding appearance of sponges at different time points during the degradation process using 5 U/mL collagenase at 37 °C for 24 h. It can be seen that the pure collagen sponge could barely resist the enzymatic degradation that had lost its shape at the beginning and been almost totally degraded after 24 h. On the contrary, the epoxides cross-linked sponges had survived the enzymatic degradation much better, lost much less weight and remained their intact shapes. The Col-Ep2L, who had the lowest cross-linking degrees, had still maintained its structural integrity even been degraded more than 60%. In the case of maintaining basic shapes, the degradation

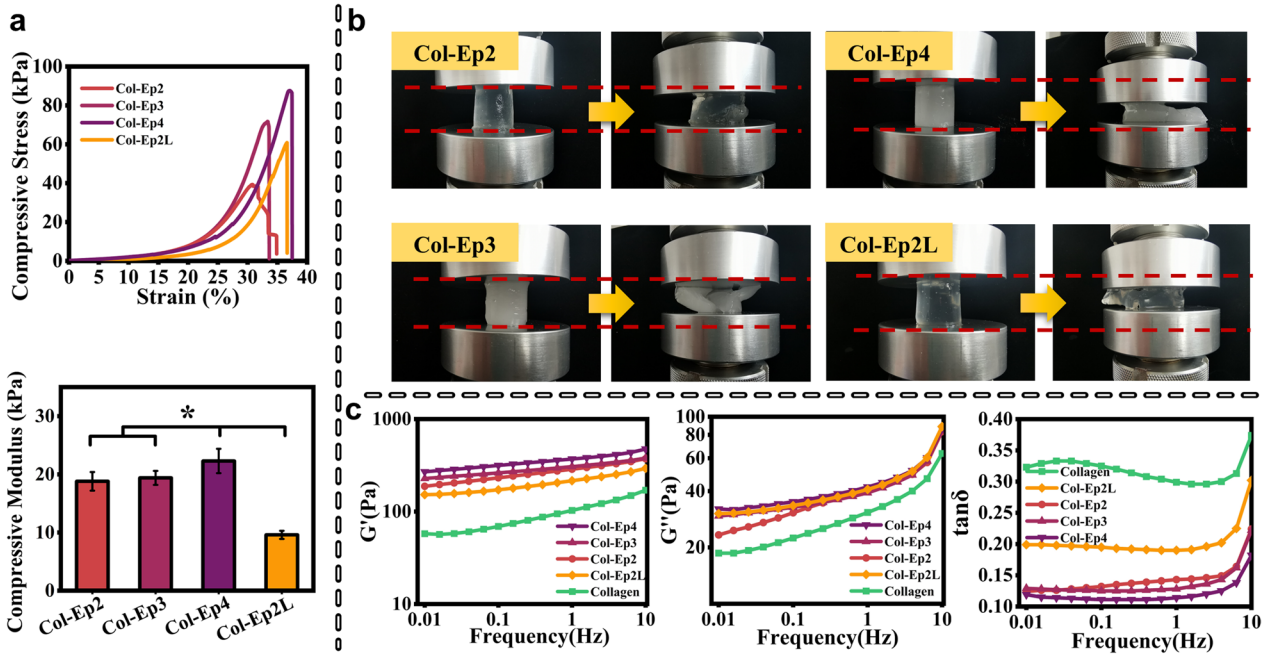


Fig. 5 **a** The compressive stress–strain curves of the gel-like precursors and the corresponding compressive modulus evaluated by the range of stress–strain curves from 0 to 40% with $R^2 > 0.99$; **b** The appearance of the gel-like precursors during the compressive test; **c** The rheological properties of the gel-like precursors, including the storage modulus (G′), loss modulus (G″), and the loss tangent (tan δ)

rates among different sponges had considerable difference, as shown in Fig. 6, that only less than 25% of Col-Ep4 was degraded while only about 30% of Col-Ep2L was residual. These results demonstrated that the multi-functional epoxides could achieve the regulation of enzymatic degradation rates of cross-linked sponges, which provided a hope to meet the practical requirements flexibly as the tissue engineering scaffolds. For instance, it would be better for the collagen-based scaffolds used for skins to be degraded and absorbed in a few weeks [41], yet when it came to bone-related applications, the scaffolds would be expected to last for months [42].

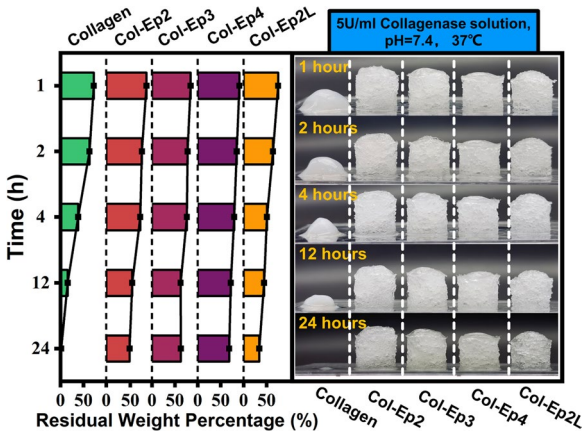


Fig. 6 The residual weight percentages and appearance of cross-linked sponges at each time point, the degradation was processed in 5 U/mL type I collagenase in PBS solutions (pH = 7.4) at 37 °C for 24 h

The degree of cross-linking could provide the theoretical basis to illuminate the difference in structures, stabilities, and mechanical behaviors among sponges. The thermal stability, which was represented as shrinkage temperatures or denaturation temperatures according to the state of collagen-based materials, was a direct and convenient way to estimate the cross-linking degrees [24]. Figure 7a had shown the denaturation temperatures of sponges, and the untreated collagen was added as reference. It can be seen that the denaturation temperature of Col-Ep4 could reach 75.3 °C, and Col-Ep3, Col-Ep2, and Col-Ep2L were gradually lower to 69.9 °C, 68.4 °C and 66.4 °C respectively, which were all higher than 63.9 °C of pure collagen. Corresponding to the structures of epoxides, it can be seen that the higher functionalities of epoxides could bring higher denaturation temperatures to sponges, which also suggested the higher cross-linking degrees. Meanwhile, the lower denaturation temperature of Col-Ep2L than Col-Ep2 demonstrated that the longer backbone of epoxides would lead to the rather low cross-linking degrees, which was agreed with the deductions based on the morphologies of sponges

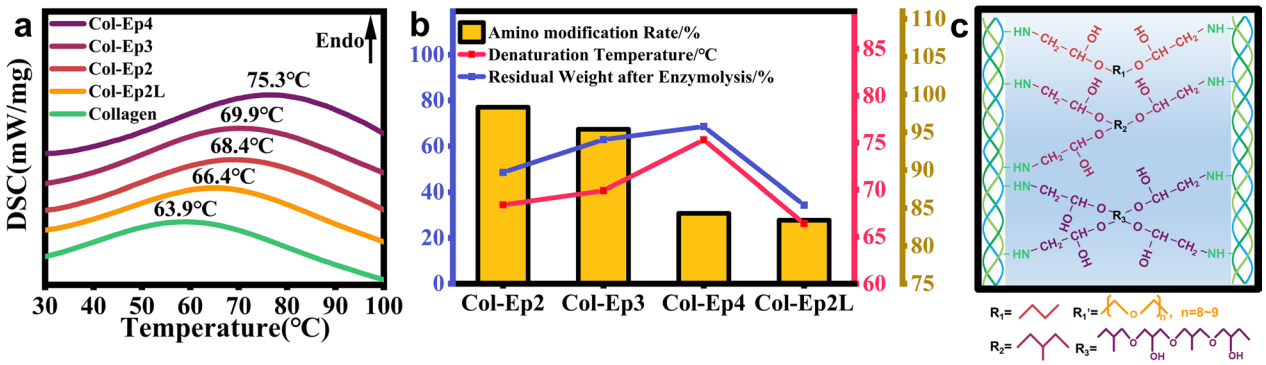


Fig. 7 **a** The DSC curves of sponges; **b** The amino modification rates of sponges measured by TNBS assay and corresponding denaturation temperatures and residual weights after enzymolysis; **c** The reaction scheme between epoxides and collagen molecules

and swelling behaviors of gel-like precursors. Combined with the structures of sponges, the higher cross-linking degrees could establish the tighter cross-linking connections among collagen molecules and form the compacter cross-linking networks in sponges, reflected as the stronger ability to resist thermal or enzymatic degradations.

The TNBS assay was introduced to evaluate the consumption of amino groups on the side chains of collagen molecules, which was also a common approach to quantify the cross-linking degrees. The results were shown in Fig. 7b and corresponding denaturation temperatures and enzyme degradation degrees were used for comparison, and the reaction scheme was presented in Fig. 7c. It was interesting to find that the consumption rates of amino groups were not fit with cross-linking degrees expressed as stabilities. The modification rates of Col-Ep2L and Col-Ep4 were similar; they were also the weakest and strongest sponges, respectively, according to the mechanical properties, thermal and enzymatic stabilities.

It was obvious that the actual cross-linking degrees depended on the functionalities and backbone lengths of multi-functional epoxides. The similarity between the epoxides used for Col-Ep2L and Col-Ep4 was the rather long backbones, while the backbone lengths of epoxides used for Col-Ep2 and Col-Ep3 were closer. The corresponding was that the amino modification rates of Col-Ep2L and Col-Ep4 were lower than that of Col-Ep2 and Col-Ep3, which indicated that the long backbones of epoxides would reduce the binding amounts of epoxide molecules with collagen molecules. However, the higher functionalities could made up the disadvantages brought by long backbones, as assumed by the swelling behaviors of gel-like precursors. Although the backbone lengths of epoxides used for Col-Ep2, Col-Ep3, and Col-Ep4 were gradually increased, the denaturation temperatures among corresponding sponges were also increasing along with the increasing functionalities of epoxides. In fact, there were both grafting and cross-linking reactions that happened between collagen molecules and epoxide molecules, that the epoxide molecules had to grafted to collagen molecules first and then linked to the other collagen molecules and formed effective cross-linking bonds. It could be speculated that there were only a certain proportion of grafted cross-linker molecules that could further cross-link to the other collagen molecules, so that the higher binding amount could provide more chances to form cross-linking bonds. The long backbones of epoxide used for Col-Ep2L had reduced the binding amount according to the results of TNBS, so that the final effective cross-linking bonds were less than that of Col-Ep2 and led to relatively weaker performance of Col-Ep2L. As for Col-Ep4, the higher functionalities of epoxides had provided much more chance to form cross-linking bonds and improved the proportion of cross-linked epoxide molecules, so that Col-Ep4 had the highest cross-linking degrees and best performances.

3.4 In vitro cytocompatibility evaluations

The quantitatively analysis of the proliferation of L929 fibroblasts based on the CCK-8 method was used to evaluate the in vitro cytocompatibility of sponges and exhibited as the OD values at day1, day3, and day5 in Fig. 8a. The gradually increased OD values demonstrated that the good promotions of sponges to cell proliferation that affirmed the basic security to be utilized in biomedical occasions.

The CLSM images after 3 days culturing of L929 fibroblasts were shown in Fig. 8b and it can be seen that the number of cells of Col-Ep2 and Col-Ep2L were higher, while Col-Ep3 and Col-Ep4 had attached much fewer cells. It can be assumed that the high cross-linking degrees might lead to the stronger mask effects of epoxide molecules on the RGD sequence of collagen

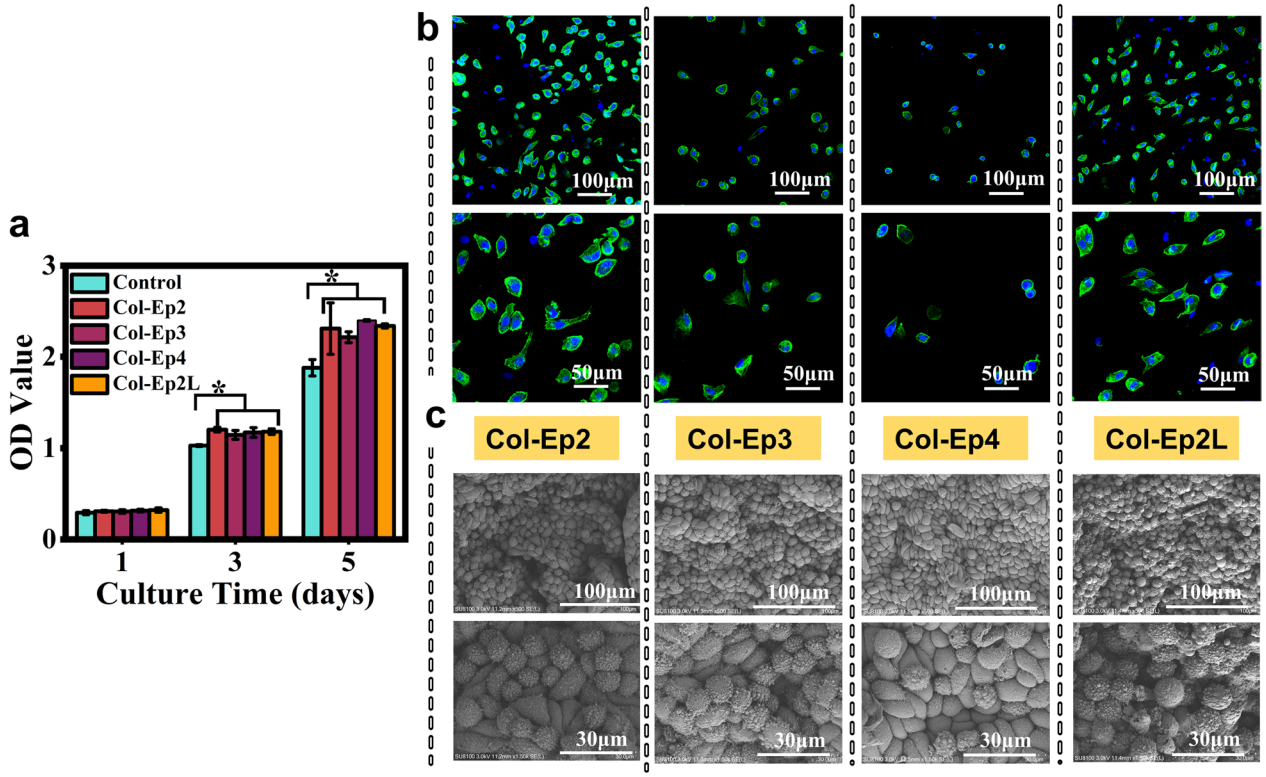


Fig. 8 **a** The optical density of cells measured by CCK-8 method to evaluate the proliferation of L929 fibroblast cultured on the cross sections of sponges. The values were presented as mean ± standard deviation (SD) for six parallel wells; **b** The cell morphologies observed by CLSM based on L929 fibroblasts, who had been seeded on the cross-sections of sponges after 3 days and stained by rhodamine-phalloidin, scale bar: 100 μm at first line, 50 μm at second line; **c** The SEM images of L929 fibroblast cultured on the cross-sections of cross-linked sponges after 5 days, scale bar: 100 μm at first line, 30 μm at second line

molecules for fibroblasts to identify. Besides, the more intensive porous structure of Col-Ep3 and Col-Ep4 shown in Fig. 1 might reduce the available physical adhesion sites for cells. Still, after 5 days culturing, the SEM images in Fig. 8c had confirmed that the reduced number of cells could be barely associated to any potential cytotoxicity, considering that the fibroblasts had fully covered the seeding areas of all sponges. Furthermore, the cells attached to the sponges had the filling shapes and many of them had covered with vesicle, which promoted the normal metabolism and function of fibroblasts [43].

4 Conclusion

In present work, the multi-functional epoxides were applied on collagen to establish the collagen sponges for tissue engineering. With the help of the functionalities and backbone lengths of epoxides, the sponges had achieved the tunable porous structure with wide ranges of pore sizes and the versatile mechanical properties and stabilities. The present works also verified the direct relations of the microstructure and mechanical behaviors between the gel-like precursors and final sponges. Furthermore, the sponges had the similar in vitro cytocompatibility that ensure the maximum general applicability for tissues engineering. Overall, the potentials and theoretical foundations of multi-functional epoxides have been explored and promoted from the natural animal tissues to the artificial collagen-based biomaterials, which were also given great hopes to help the development of customizable collagen-based biomaterials for wider fields.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (Nos. 22078206). The authors would thank to Qingshuang Song (College of Light Industry, Textile and Food Engineering, Sichuan University) for her help in DSC test. We would also be grateful to Doctor Hui Wang (Analytical & Testing Center, Sichuan University) for her help of taking SEM images.

Author contributions

GYL designed this project and revised the manuscript. YZZ performed experiments, analyzed data, draw the scheme, and wrote the manuscript. CKY and MG performed experiments. XXZ and XQZ reviewed the manuscript. All authors read and approved the final manuscript.

Funding
This research was financially supported by the National Natural Science Foundation of China (No. 22078206).

Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Guoying Li is a member of the editorial board of Collagen and Leather, and was not involved in the editorial review, or the decision to publish this article. All authors declare that there are no competing interests.

Author details
[1]National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, People's Republic of China. [2]Key Laboratory of Leather Chemistry and Engineering (Sichuan University), Ministry of Education, Chengdu 610065, People's Republic of China. [3]Max Planck Institute for Polymer Research, 55128 Mainz, Germany.

Received: 9 May 2023 Revised: 25 September 2023 Accepted: 5 October 2023
Published online: 12 October 2023

References

1. Bhatnagar MS. Epoxy resins from 1980 to date. Part 1. Polym Plast Technol Eng. 2006;32:53–113. https://doi.org/10.1080/03602559308020155.
2. Fu H, Huang H, Wang Q, Zhang H, Chen H. Properties of aqueous polyurethane dispersion modified by epoxide resin and their use as adhesive. J Dispers Sci Technol. 2009;30:634–8. https://doi.org/10.1080/01932690802598507.
3. Dewle A, Pathak N, Rakshasmare P, Srivastava A. Multifarious fabrication approaches of producing aligned collagen scaffolds for tissue engineering applications. ACS Biomater Sci Eng. 2020;6:779–97. https://doi.org/10.1021/acsbiomaterials.9b01225.
4. Bloebaum RD, Wilson AS, Martin WN. A review of the collagen orientation in the articular cartilage. Cartilage. 2021;13:367S-S374. https://doi.org/10.1177/1947603520988770.
5. Gu L, Shan T, Ma YX, Tay FR, Niu L. Novel biomedical applications of crosslinked collagen. Trends Biotechnol. 2019;37:464–91. https://doi.org/10.1016/j.tibtech.2018.10.007.
6. Fertala A. Three decades of research on recombinant collagens: Reinventing the wheel or developing new biomedical products? Bioengineering (Basel). 2020. https://doi.org/10.3390/bioengineering7040155.
7. Harris FEM. Tanning studies with epoxy resins. Preliminary evaluation of the leather. JALCA. 1956;51:574–83.
8. Sykes RL. The cross-linking of collagen with aliphatic epoxides. J Soc Leather Technol Chem. 1957;41:199–206.
9. Delgado LM, Fuller K, Zeugolis DI. Influence of cross-linking method and disinfection/sterilization treatment on the structural, biophysical, biochemical, and biological properties of collagen-based devices. ACS Biomater Sci Eng. 2018;4:2739–47. https://doi.org/10.1021/acsbiomaterials.8b00052.
10. Tu R, Shen SH, Lin D, Hata C, Thyagarajan K, et al. Fixation of bioprosthetic tissues with monofunctional and multifunctional polyepoxy compounds. J Biomed Mater Res. 1994;28:677–84. https://doi.org/10.1002/jbm.820280604.
11. Doillon CFWCJ, Brandwein S, Silver FH. Collagen-based wound dressings: control of the pore structure and morphology. J Biomed Mater Res. 1986;20:1219–28.
12. Lee JM, Pereira CA, Kan LWK. Effect of molecular structure of poly(glycidyl ether) reagents on crosslinking and mechanical properties of bovine pericardial xenograft materials. J Biomed Mater Res. 1994;28:981–92. https://doi.org/10.1002/jbm.820280903.
13. Lohrr JM, Budig L, Sagartz J, Girida S, Thyagarajan K, Tu R. Evaluation of two epoxy ether compounds for biocompatible potential. Thoughts Prog. 1992;16:630–3. https://doi.org/10.1111/j.1525-1594.1992.tb00563.x.
14. Zhuravleva IY, Karpova EV, Oparina LA, Poveschenko OV, Surovtseva MA, Titov AT, Ksenofontov AL, Vasilieva MB, Kuznetsova EV, Bogachev-Prokophiev AV, Trofimov BA. Cross-linking method using pentaepoxide for improving bovine and porcine bioprosthetic pericardia: a multiparametric assessment study. Mater Sci Eng C Mater Biol Appl. 2021;118:111473. https://doi.org/10.1016/j.msec.2020.111473.
15. Di Y, Heath RJ. Collagen stabilization and modification using a polyepoxide, triglycidyl isocyanurate. Polym Degrad Stab. 2009;94:1684–92. https://doi.org/10.1016/j.polymdegradstab.2009.06.019.
16. Bock N, Riminucci A, Dionigi C, Russo A, Tampieri A, Landi E, Goranov VA, Marcacci M, Dediu V. A novel route in bone tissue engineering: magnetic biomimetic scaffolds. Acta Biomater. 2010;6:786–96. https://doi.org/10.1016/j.actbio.2009.09.017.
17. Zheng X, Chen Y, Dan N, Dan W, Li Z. Highly stable collagen scaffolds crosslinked with an epoxidized natural polysaccharide for wound healing. Int J Biol Macromol. 2021;182:1994–2002. https://doi.org/10.1016/j.ijbiomac.2021.05.189.
18. Minardi S, Corradetti B, Taraballi F, Sandri M, Van Eps J, Cabrera FJ, Weiner BK, Tampieri A, Tasciotti E. Evaluation of the osteoinductive potential of a bio-inspired scaffold mimicking the osteogenic niche for bone augmentation. Biomaterials. 2015;62:128–37. https://doi.org/10.1016/j.biomaterials.2015.05.011.
19. Zeeman R, Dijkstra PJ, van Wachem PB, van Luyn MJA, Hendriks M, Cahalan PT, Feijen J. The kinetics of 1,4-butanediol diglycidyl ether crosslinking of dermal sheep collagen. J Biomed Mater Res. 1999;54:541–8.
20. Zhang J, Ma X, Fan D, Zhu C, Deng J, Hui J, Ma P. Synthesis and characterization of hyaluronic acid/human-like collagen hydrogels. Mater Sci Eng C Mater Biol Appl. 2014;43:547–54. https://doi.org/10.1016/j.msec.2014.07.058.
21. Kim HJ, Kim KK, Park IK, Choi BS, Kim JH, Kim MS. Hybrid scaffolds composed of hyaluronic acid and collagen for cartilage regeneration. Tissue Eng Regen Med. 2012;9:57–62. https://doi.org/10.1007/s13770-012-0007-7.
22. Trimbach DC, Keller B, Bhat R, Zankovych S, Pöhlmann R, Schröter S, Bossert J, Jandt KD. Enhanced osteoblast adhesion to epoxide-functionalized surfaces. Adv Funct Mater. 2008;18:1723–31. https://doi.org/10.1002/adfm.200701491.
23. Zhang Y, Yang C, Yin S, Zhang X, Peng X, Li G. Exploration of 2D and 2.5D conformational designs applied on epoxide/collagen-based integrative biointerfaces with device/tissue heterogeneous affinity. ACS Appl Mater Interfaces. 2023;15:22876–91. https://doi.org/10.1021/acsami.3c00945.
24. Sung HW, Hsu HL, Shih CC, Lin DS. Cross-linking characteristics of biological tissues fixed with monofunctional or multifunctional epoxy compounds. Biomaterids. 1996;17:1405–10. https://doi.org/10.1016/0142-9612(96)87282-6.
25. Sung HW, Hsu CS, Wang SP, Hsu HL. Degradation potential of biological tissues fixed with various fixatives: an in vitro study. J Biomed Mater Res. 1997;35:147–55. https://doi.org/10.1002/(SICI)1097-4636(199705)35:23.0.CO;2-N.
26. Yang H, Shen L, Bu H, Li G. Stable and biocompatible hydrogel composites based on collagen and dialdehyde carboxymethyl cellulose in a biphasic solvent system. Carbohydr Polym. 2019;222:114974. https://doi.org/10.1016/j.carbpol.2019.114974.
27. Zhang F, Wang C, Mu C, Lin W. A novel hydrophobic all-biomass aerogel reinforced by dialdehyde carboxymethyl cellulose for oil/organic solvent-water separation. Polymer. 2022. https://doi.org/10.1016/j.polymer.2021.124402.
28. Meyer M. Processing of collagen based biomaterials and the resulting materials properties. Biomed Eng. 2019;18:24. https://doi.org/10.1186/s12938-019-0647-0.
29. Davidenko N, Gibb T, Schuster C, Best SM, Campbell JJ, Watson CJ, Cameron RE. Biomimetic collagen scaffolds with anisotropic pore architecture. Acta Biomater. 2012;8:667–76. https://doi.org/10.1016/j.actbio.2011.09.033.

30. Xia Z, Villa MM, Wei M. A biomimetic collagen-apatite scaffold with a multi-level lamellar structure for bone tissue engineering. J Mater Chem B. 2014;2:1998–2007. https://doi.org/10.1039/C3TB21595D.
31. Bose S, Roy M, Bandyopadhyay A. Recent advances in bone tissue engineering scaffolds. Trends Biotechnol. 2012;30:546–54. https://doi.org/10.1016/j.tibtech.2012.07.005.
32. Zhang Y, Zhang M, Cheng D, Xu S, Du C, Xie L, Zhao W. Applications of electrospun scaffolds with enlarged pores in tissue engineering. Biomater Sci. 2022;10:1423–47. https://doi.org/10.1039/d1bm01651b.
33. Jenkins D, Salhadar K, Ashby G, Mishra A, Cheshire J, Beltran F, Grunlan M, Andrieux S, Stubenrauch C, Cosgriff-Hernandez E. PoreScript: semi-automated pore size algorithm for scaffold characterization. Bioact Mater. 2022;13:1–8. https://doi.org/10.1016/j.bioactmat.2021.11.006.
34. Zhao S, Malfait WJ, Guerrero-Alburquerque N, Koebel MM, Nystrom G. Biopolymer aerogels and foams: chemistry, properties, and applications. Angew Chem Int Ed Engl. 2018;57:7580–608. https://doi.org/10.1002/anie.201709014.
35. Yuichiro. Mechanical properties and immunogenicity of vascular xenografts. Jpn J Surg. 1991;21:533–41.
36. Sung HW, Hsu HL, Shih CC, Lin DS. Cross-linking characteristics of biological tissues fixed with monofunctional or multifunctional epoxy compounds. Biomaterials. 1996;17:1405–10. https://doi.org/10.1016/0142-9612(96)87282-6.
37. Zhang L, Zhang Y, Jiang B, Fan HS, Zhang XD. Characterization of an insoluble collagen sponge and the potential for tissue engineering scaffold. Mater Sci Forum. 2009;610–613:1378–81. https://doi.org/10.4028/www.scientific.net/MSF.610-613.1378.
38. Ghodbane SA, Dunn MG. Physical and mechanical properties of cross-linked type I collagen scaffolds derived from bovine, porcine, and ovine tendons. J Biomed Mater Res A. 2016;104:2685–92. https://doi.org/10.1002/jbm.a.35813.
39. Ryan AJ, O'Brien FJ. Insoluble elastin reduces collagen scaffold stiffness, improves viscoelastic properties, and induces a contractile phenotype in smooth muscle cells. Biomaterials. 2015;73:296–307. https://doi.org/10.1016/j.biomaterials.2015.09.003.
40. Kanungo BP, Silva E, Van Vliet K, Gibson LJ. Characterization of mineralized collagen-glycosaminoglycan scaffolds for bone regeneration. Acta Biomater. 2008;4:490–503. https://doi.org/10.1016/j.actbio.2008.01.003.
41. Li H, Chen X, Ren K, Wu L, Chen G, Xu L. Qualitative study on diabetic cutaneous wound healing with radiation crosslinked bilayer collagen scaffold in rat model. Sci Rep. 2023;13:6399. https://doi.org/10.1038/s41598-023-33372-z.
42. Xu J, Fahmy-Garcia S, Wesdorp MA, Kops N, Forte L, De Luca C, Misciagna MM, Dolcini L, Filardo G, Labberte M, Vancikova K, Kok J, van Rietbergen B, Nickel J, Farrell E, Brama PAJ, van Osch G. Effectiveness of BMP-2 and PDGF-BB adsorption onto a collagen/collagen-magnesium-hydroxyapatite scaffold in weight-bearing and non-weight-bearing osteochondral defect bone repair: in vitro, ex vivo and in vivo evaluation. J Funct Biomater. 2023;10:10. https://doi.org/10.3390/jfb14020111.
43. Muñoz-Ruíz A, Escobar-García DM, Quintana M, Pozos-Guillén A, Flores H. Synthesis and characterization of a new collagen-alginate aerogel for tissue engineering. J Nanomater. 2019;2019:1–10. https://doi.org/10.1155/2019/2875375.

Publisher's Note