PART I

1

COLLAGEN-BASED POROUS SCAFFOLDS FOR TISSUE ENGINEERING

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1.1 INTRODUCTION

Collagen is one of the main components of extracellular matrices that provide mechanical support and biological signals to cells for cellular activities [1]. Collagen has attracted wide attention for biomedical applications because of its versatile property [2–5]. It has been used to construct scaffolds in different forms either with or without hybridization with other biodegradable synthetic or naturally derived materials for tissue engineering. Collagen-based porous scaffolds have been developed through many methods and widely used for tissue engineering of a variety of tissues and organs such as skin [3], bone [5], cartilage [6], ligament [7], blood vessel [8] and nerve [9]. Their pore structures have been well designed and controlled to meet the requirements for cell distribution and cell interaction to promote functional tissue regeneration [10–15]. Hybridization of collagen with mechanically strong synthetic polymers has also developed to improve its mechanical property. Some of the latest developments of collagen-based scaffolds with controlled pore structures and composite structures are summarized and highlighted in this chapter.

Biomaterials from Nature for Advanced Devices and Therapies, First Edition. Edited by Nuno M. Neves and Rui L. Reis.

1.2 COLLAGEN SPONGES

Collagen is a water-soluble polymer and is very easy to prepare its porous sponge by using freeze-drying method [16]. Collagen aqueous solution or collagen gel can be frozen at a low temperature, subsequently freeze-dried at a low pressure and finally cross-linked to prepare collagen porous sponges. During freezing process, freezing temperature may affect the formation and growth of ice crystals in the aqueous solution. Therefore, controlling of freezing temperature has been used to control the porous structure of collagen sponges. Fast freezing at a lower temperature induces cracking, uniform small channels and formation of a fibrous structure. Slow freezing at a higher temperature results in nonuniformity and large pores with more collapsed pores than continuous channels. A unidirectional freezing-drying method has been developed to prepare unidirectionally structured collagen sponge [17]. Collagen sponge resembling the extracellular matrix structure of a particular tissue has been prepared by specific freezing regimes [18]. Although some methods have been developed to prepare collagen sponges with partially controlled pore structures, it has been pursued by many researchers to make the sponge pore open and increase the interconnectivity. Recently a method by using embossing ice particulates as a temperature to precisely control the pore structure of collagen sponges has been developed [10].

The preparation scheme using embossing ice particulates is shown in Figure 1.1. At first, water droplets are prepared by spraying pure water on the surface of a hydrophobic film and water droplets are formed on the surface. The size of the water droplets can be controlled by spraying condition such as spraying speed and spraying time. Or the water droplet can be printed on the hydrophobic surface by a dispenser and the size of the ice droplets can be controlled by the volume of injected volume of water. Subsequently, the water droplets are frozen at a low temperature to form

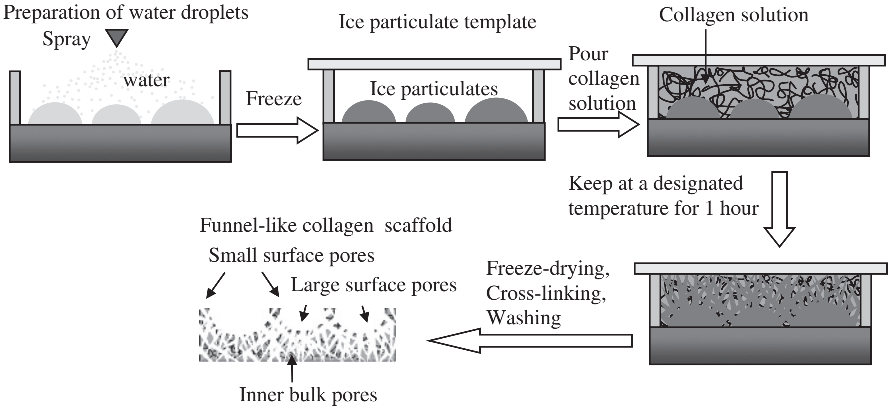

FIGURE 1.1 Preparation scheme of the funnel-like collagen sponge using embossing ice particulates. Adapted and reproduced from Ref. 10 (DOI: 10.1177/0883911510370002). For a color version of this figure, see the color plate section.

ice particulates embossing the membrane surface. The size and density of embossing ice particulates are controllable. Finally, collagen aqueous solution is eluted onto the embossing ice particulates, frozen, freeze-dried and cross-linked to prepare collagen sponges with a controlled pore structure. Usually the temperature of ice particulates and collagen aqueous solution should be balanced before eluting collagen aqueous solution onto the ice particulates. The prepared collagen porous sponges have large open pores on the surface and interconnected bulk pores underlying the large surface pores. Such structure is very similar to a funnel and therefore the collagen sponges prepared by this method are referred as funnel-like collagen sponges.

The photo of funnel-like collagen sponge prepared with 398 μm-diameter ice particulates and 1.0% collagen aqueous solution shows clear large pores are evenly distributed on the surface of the collagen sponge (Fig. 1.2a). Scanning electron microscopy images show that large pores are formed on the top surface and interconnected bulk pores are formed beneath the surface pores (Fig. 1.2b and c). The mean diameter of the large surface pores is almost the same as that of the embossing

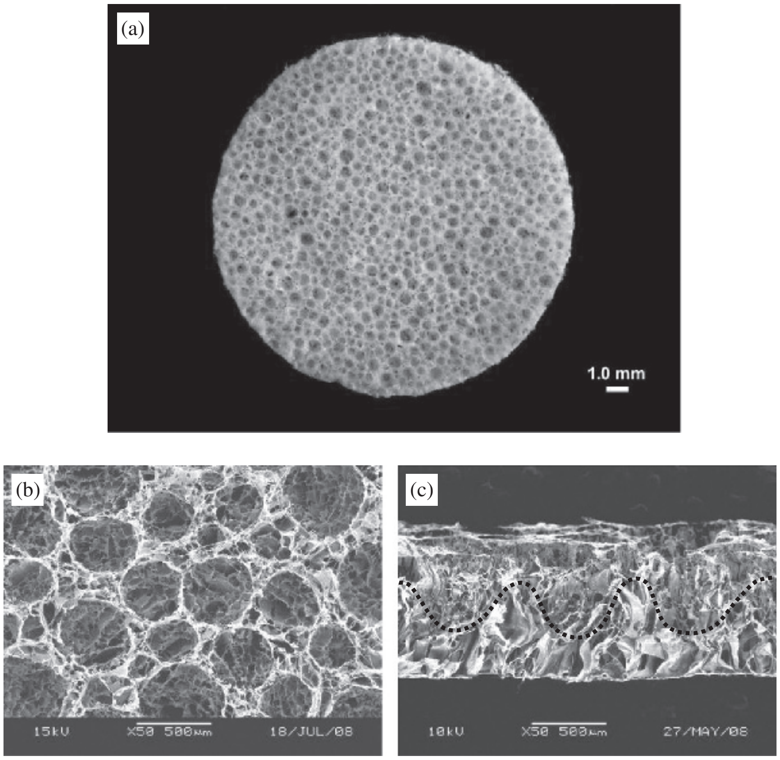

FIGURE 1.2 Photograph (a) and SEM photomicrographs (b, c) of top surface (b) and cross-section (c) of funnel-like collagen sponge prepared with 398 μm-diameter ice particulate template at –3°C. Adapted and reproduced from Ref. 10 (DOI: 10.1177/0883911510370002). For a color version of this figure, see the color plate section.

ice particulates which are used as templates because the large surface pores should be the replicas of the embossing ice particulates.

The underlying bulk pores are interconnected with the large surface pores and extend into the bulk body of the sponge from the surface pores. The underlying bulk pores are the replicas of the ice crystals that are formed during freeze-drying. Therefore, the pore structure of the funnel-like collagen sponges is mainly dependent on the size and density of embossing ice particulates and the freezing temperature. The size and density of surface large pores are determined by the size and density of embossing ice particulates. The size and interconnectivity of underlying bulk pores are dependent on the freezing temperature. Funnel-like collagen sponges prepared with the same size of ice particulates (398 μm) but four different freezing temperatures have the same surface large pores (Fig. 1.3a–d). However, the underlying bulk pores have different size. The size of the bulk pores decreases with a decrease of freezing temperature. Figure 1.3e shows the speculated schematic diagram of the formation of the ice crystals during the freezing process and the effect of temperature on the formation of the ice crystals. When the temperature of the aqueous collagen solution is lowered to its freezing point in the absence of embossing ice particulates, random flake-like ice crystals are formed. Some ice crystals may start to form on the surface while some may start from the inner bulk solution. The connectivity of these ice crystals is low. Therefore, the collagen sponges prepared without embossing ice templates have a random porous structure and low pore interconnectivity. In contrast, when the ice particulate templates are used, the ice particulates can serve as nuclei to initiate ice crystallization at the freezing interface of the liquid phase collagen solution. The newly formed ice particulates gradually grow into connected dendritic network. The embossing ice particulates and the newly formed dendritic ice crystal network should result in the formation of the unique funnel-like porous structure of the collagen sponges. Formation of the new ice crystals during freezing process is affected by the temperature. Low temperature results in quick formation of dense, small ice crystals and therefore formation of small bulk pores. On the other hand, high temperature results in slow formation of sparse, big ice crystals and formation of large bulk pores.

The funnel-like porous structure facilitates cell seeding and homogeneous cell distribution in the collagen sponges. Compared to control collagen sponge prepared at –3°C without ice template, the funnel-like collagen sponge prepared with 398 μm-diameter embossing ice particulates at –3°C shows good cell penetration and spatially more homogeneous distribution [10]. The funnel-like collagen sponges have been used for three-dimensional culture of fibroblasts and chondrocytes for dermal and cartilage tissue engineering, respectively [19].

The embossing ice particulates method can also be used for preparation of porous scaffolds of other materials such as gelatin, chitosan and hyaluronic acid as long as the freezing temperature of the solution of the other materials is not higher than the melting temperature of the ice particulates. Funnel-like porous scaffolds of chitosan, hyaluronic acid and hyaluronic acid/collagen composite are prepared by the method and have the same effect on cell seeding and distribution as that of funnel-like collagen sponges [20–22].

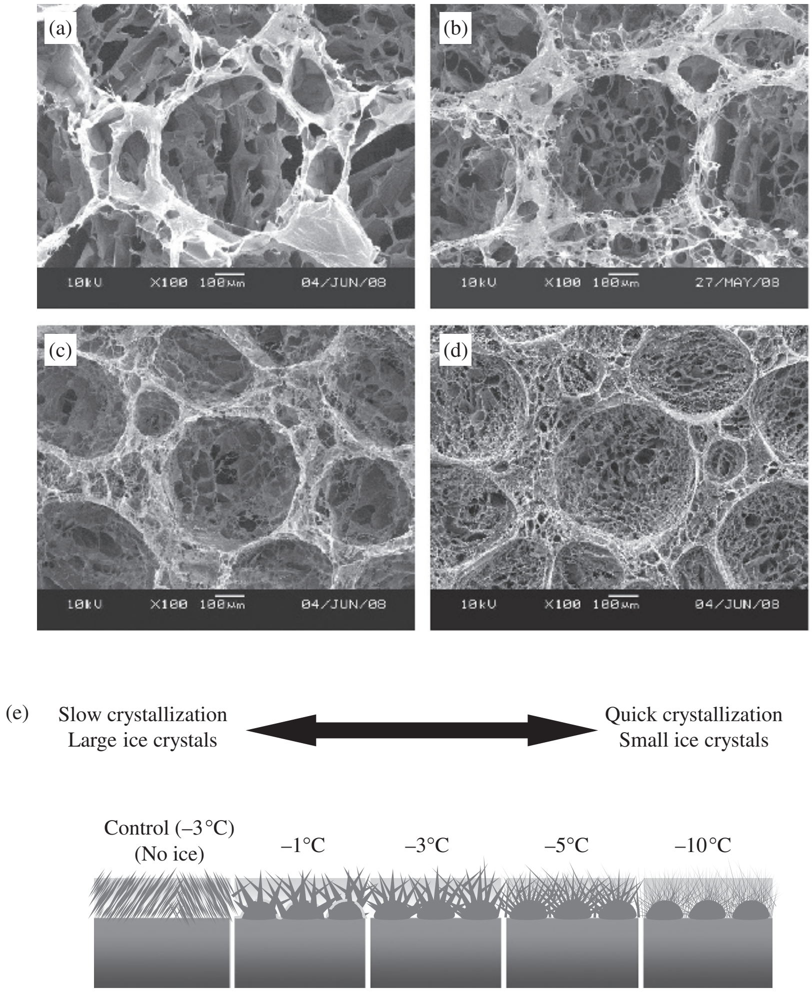

FIGURE 1.3 SEM photomicrographs of top surfaces of funnel-like collagen sponges prepared by using the embossing ice particulate template with ice particulate diameter of 398 μm at different temperature of –1 (a), –3 (b), –5 (c) and –10°C (d). Schematic diagram of the effect of temperature on the formation of new ice crystals on the ice particulate template and formation of new ice crystals on the surface without ice particulates (e). Adapted and reproduced from Ref. 10 (DOI: 10.1177/0883911510370002).

1.3 COLLAGEN SPONGES WITH MICROPATTERNED PORE STRUCTURES

Micropattern structures of porous scaffolds are important to guide the regeneration of tissues and organs with complex structures. The micropatterned structures can be micropatterned pores or micropatterned bioactive molecules. They can arrange cells into a predesigned location and guide the regeneration of complex networks such as capillary and neuronal networks in accordance with the micropatterns [23, 24]. Micropatterned structures in three-dimensional (3D) porous scaffolds are desirable because they can mimic the same biological and physiochemical cues as those present in the *in vivo* microenvironments that surround cells. The embossing ice particulates method can be used to prepare such porous scaffolds with micropatterned structures [25].

Template of micropatterned ice particulates or ice lines is used to prepare the micropattern-structure collagen sponges. The ice particulate and ice line templates are prepared by ejecting water droplets through a dispensing machine on a film at a low temperature. The micropatterns of the ice particulates and ice lines can be designed using a computer program. The other procedures are the same as the embossing ice particulates method as above described. Figure 1.4 shows four types of micropatterned ice templates that are prepared by designing the micropattern and size of the ice particulates (Figure 1.4a, c, e and g). Collagen sponges with different micropatterned pore structures are prepared by using the micropatterned ice particulates as templates (Figure 1.4b, d, f and h). The micropatterned pore structures are the negative replica of the ice templates. The other pores surrounding and underlying the micropatterned pores and lines are negative replica of ice crystals generated during the freezing process.

The micropatterned pore layer can be stacked to construct collagen sponges with 3D micropatterned pores (Figure 1.4i and j). In this case, the frozen collagen solution on the first layer of micropatterned ice particulates should be used to prepare the second layer of micropatterned ice particulates instead of the film. By repeating the procedure and later following it with the freeze-drying, cross-linking and washing processes, collagen sponges with micropatterned 3D structures can be prepared. The 3D micropatterned collagen sponge has its top surface similar to that of the collagen sponge with one layer of micropatterned structure as shown in Figure 1.4b. The cross-section has stacked pore structure (Figure 1.4j).

The micropatterning method can also be used to micropattern bioactive molecules in the 3D porous collagen scaffolds [11]. For incorporation of bioactive molecules, a collagen aqueous solution containing bioactive molecules other than pure water should be used. Bioactive molecules are mixed with the collagen aqueous solution. The mixture solution is ejected onto the low-temperature film through a nozzle using a dispensing machine. Different micropatterns composed of the collagen/bioactive molecule solutions can be prepared by designing a program. The ice micropatterns of bioactive molecules are used to prepare collagen porous scaffolds with micropatterned bioactive molecules. The other preparation procedures are the same as above described. The bioactive molecules can also be 3D micropatterned in collagen sponges by repeating the above-mentioned micropatterning procedure. The stacking method is the same as that of collagen sponges with 3D micropatterned pores. Not only single bioactive molecules but also multibioactive molecules can be co-micropatterned in collagen sponges. The multibioactive molecules can be mixed and co-micropatterned together or the multibioactive molecules can be micropatterned separately to construct a co-micropattern structure.

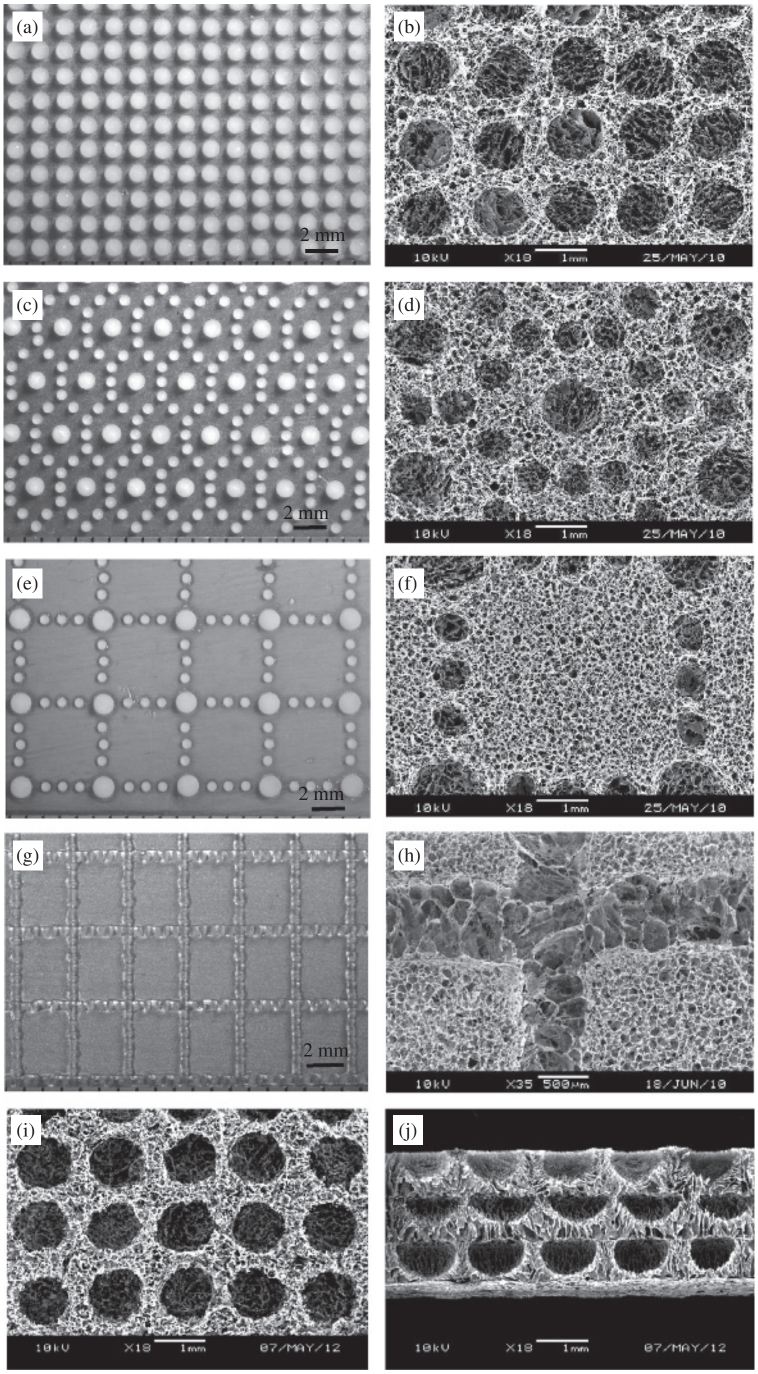

FIGURE 1.4 Photomicrographs of four types of micropatterned ice particulates templates (a, c, e and g) and SEM Photomicrographs collagen porous scaffolds prepared with the micropatterned templates (b, d, f and h) and collagen sponge with three-dimensionally micropatterned pores that is prepared with micropatterned templates shown in a (i: top surface, j: cross section). Adapted and reproduced with permission from Ref. 25 (DOI: 10.1002/adma.201200237).

1.4 COLLAGEN SPONGES WITH CONTROLLED BULK STRUCTURES

Although funnel-like collagen sponges prepared with embossing ice particulates have unique pore structures for easy cell seeding and cell penetration to underlying pores, obtaining a homogeneous cell distribution in thick and large scaffolds remain challengeable. To control the bulk pore structures of large and thick scaffolds, some preparation methods have been developed [26, 27]. Among these methods, the porogen-leaching method offers many advantages for the easy manipulation and control of pore size and porosity. Although the porogen materials can leave replica pores after leaching, they cannot initiate the formation of surrounding pores. As a result, isolated pores are formed in the scaffold, a situation which is not desirable for tissue engineering scaffolds. To improve pore interconnectivity, the porogen materials are bonded before mixing them with polymer matrix [28–30]. However, the bonded porogen materials require organic solvents for leaching of the porogen materials and the residual solvents are toxic to cells. Penetration of the polymer solution into the bonded porogen material becomes difficult if the polymer solution has a high viscosity.

Embossing ice particulates method shows advantages to control the surface pore structure and to increase the interconnectivity between the surface pores and surrounding pores [10, 22]. By taking this advantage, the ice particulates method should solve the interconnection problem of large scaffolds because the ice particulates can initiate formation of interconnecting ice crystals from their interface in aqueous solution. In this case, free ice particulates should be used as a porogen material. The pre-prepared free ice particulates not only work as porogens to control the pore size and porosity but also work as nuclei to initiate the formation of new ice crystals in the surrounding aqueous solution, therefore increasing the interconnectivity of the collagen sponges [12, 31].

Ice particulates are prepared by spraying Milli Q water into liquid nitrogen using a sprayer. The ice particulates can be sieved by sieves with different mesh pores to obtain ice particulates having a specific diameter. The sieving process should be conducted at a low-temperature to avoid melting of ice particulates. The pre-prepared ice particulates are spherical. The free ice particulates are mixed with collagen aqueous solution. The collagen aqueous solution is prepared by dissolving collagen in a solution of ethanol and acetic acid. The mixing process is conducted at a low temperature (e.g. –4°C) at which temperature the ice particulates does not melt and the collagen aqueous solution does not freeze. The two components should be well mixed to obtain an even distribution of ice particulates in the collagen aqueous solution. The mixture of ice particulates and collagen aqueous solution is further frozen at –80°C and freeze-dried and cross-linking to obtain collagen sponges with controlled bulk pore structures.

Collagen sponges prepared with free ice particulates have interconnected large pores and small pores (Fig. 1.5) [12]. The large pores are spherical and are the same size as the free ice particulates. The small pores have a random morphology and different sizes. The small pores surround the large spherical pores. The large pores are negative replicas of the pre-prepared free ice particulates, while the small ice

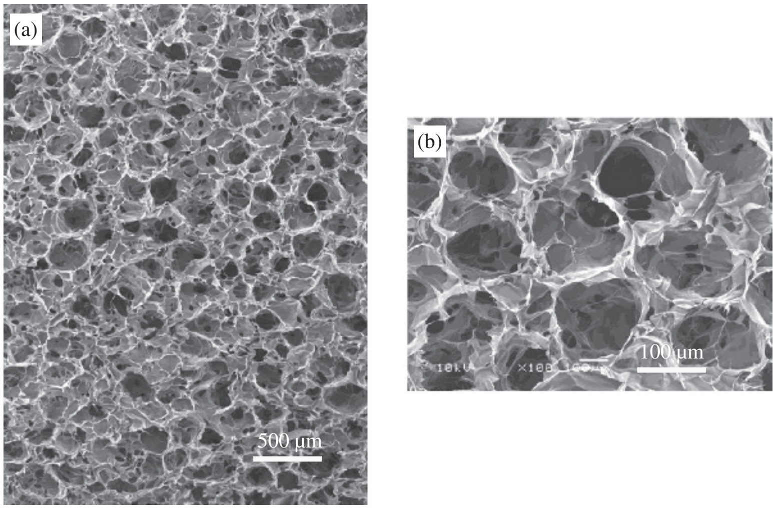

FIGURE 1.5 SEM photomicrographs of cross sections of collagen sponges prepared with 2% collagen aqueous solution and free ice particulates at a ratio of ice particulates/collagen solution of 50% at low (a) and high (b) magnification. Adapted and reproduced from Ref. 12 (DOI: 10.1177/0883911513494620).

particulates are from the ice crystals formed during freezing. The density of the large spherical pores is dependent on the percentage of free ice particulates.

Usually 2% (w/v) collagen aqueous solution is used to prepare collagen sponges when free ice particulates are used as a porogen material. Low collagen concentration (e.g. 1%) may result in collapse of some large pores due to less dense collagen matrix surrounding the large pores. High collagen concentration has very high viscosity and difficulty to be completely mixed with free ice particulates, and therefore resulting in partial collapse of the pore structure.

The concentration of collagen aqueous solution and the ratio of ice particulates can affect the mechanical property of collagen sponges. Young's modulus increases as collagen concentration increases because high collagen concentration can form dense collagen matrix surrounding the large pores. For the influence of ratio of ice particulates, there is an optimal range. When 25, 50 and 75% ice particulates are used, the Young's modulus of the collagen scaffolds increases in an order of 75% <25% <50%. The collagen sponges prepared with 50% ice particulates show the highest Young's modulus. It is much higher than that of the collagen sponge prepared without usage of ice particulates. The difference in the mechanical properties can be explained by the different pore structures. The spherical pores formed by ice particulates are thought to reinforce the collagen scaffolds. The high mechanical strength of the collagen scaffold prepared with 50% ice particulates should be due to the most appropriate packing of the large spherical pores and appropriate filling of the collagen matrix between the large spherical pores.

The collagen sponges prepared with ice particulates show a homogenous cell distribution throughout the sponges when they are used for 3D culturing of bovine articular chondrocytes. *In vivo* implantation in nude mouse shows a uniform spatial distribution of cells, a uniform ECM distribution and homogeneous tissue formation in the collagen sponges. The collagen sponges prepared with ice particulates are more favorable to the production of cartilaginous ECM and chondrocyte maturation than the collagen sponges prepared without ice particulates [31].

Collagen sponges with a gradient change of pore size from 150 μm to 500 μm are prepared by this method [32]. Ice particulates with diameters of 150–250, 250–355, 355–425 and 425–500 μm are used. Spherical large pores with diameters in a good agreement with the sizes of the ice particulates are formed in the gradient collagen sponges. The effect of pore size on cartilage tissue formation can be directly compared by culturing bovine articular chondrocytes in the gradient collagen scaffolds. The micropores in the scaffolds prepared with ice particulates in the range of 150–250 μm show the most beneficial effect on the gene expression and the production of cartilaginous matrix proteins as well as on cartilage regeneration. The good interconnectivity among the spherical large pores in the gradient collagen sponges facilitates smooth delivery of cells throughout the sponges. Cells can be smoothly delivered and homogenously distributed not only in the larger pores, but also in the small pore region. Easy filling of the seeded cells and proliferated cells in the pores in a diameter of 150–250 μm can increase the cell–cell interactions and provide a real 3D microenvironment to promote chondrogenic differentiation and cartilage tissue formation.

1.5 HYBRID SCAFFOLDS

Although collagen sponges prepared with the free ice particulates have improved mechanical property compared to collagen sponges prepared without ice particulates, their mechanical property is still inferior to porous scaffolds of biodegradable synthetic polymers. Hybrid porous scaffolds of biodegradable synthetic polymers and collagen are prepared by forming collagen microsponges or sponge in the space or opening of porous mechanical skeleton of biodegradable synthetic polymers [13–15, 33–45].

Biodegradable synthetic polymer such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymers of poly(lactic-co-glycolic acid) (PLGA) can be easily formed into porous scaffolds with designated shapes and strong mechanical property, which are the drawbacks of collagen. To combine the advantages avoiding the problems of biodegradable synthetic polymers and collagen, they have been hybridized to construct their hybrid porous scaffolds. A few types of such hybrid porous scaffolds are prepared by introducing collagen microsponges in the pores or interstices of PLGA sponges or meshes (Figure 1.6) [34, 40]. The biodegradable synthetic polymer sponge or mesh serving as a mechanical skeleton allows for easy formation into the desired shapes and provides the hybrid scaffolds with appropriate mechanical strength, while the collagen microsponges formed in pores or interstices

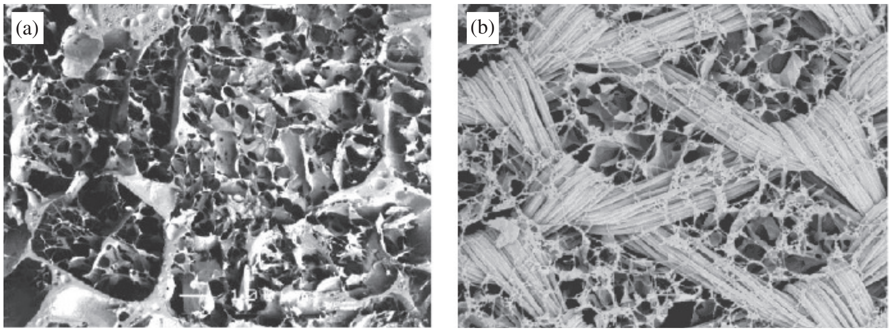

FIGURE 1.6 SEM photomicrographs of PLGA-collagen hybrid sponge (a) and PLGA-collagen hybrid mesh (b). Adapted and reproduced with permission from Ref. 34 (DOI: 10.1002/(SICI)1521-4095(200003)12:6<455::AID-ADMA455>3.0.CO;2-C) and Ref 40 (DOI: 10.1002/jbm.a.10164).

provide the hybrid scaffolds with a microporous structure, hydrophilicity and good cell interaction.

Collagen sponges can also be formed in the middle space of cup-type porous scaffolds of PLLA and PLGA to prepare hybrid porous scaffolds having high porosity and cell linkage protection capacity. In this case, the mechanical skeleton is not a simple sponge or mesh. A cup-shaped porous skeleton of biodegradable synthetic polymers is used. Collagen sponge is formed in the central space of the cup-shaped porous skeleton and collagen microsponges are formed in the pores or interstices of the porous wall of the cup skeleton [14,45]. The central collagen sponge and interstitial collagen microsponges are connected. Combination of the highly porous central collagen sponge and the surrounding PLLA-collagen sponge cup results in the high porosity of the hybrid sponge. The cup-shaped porous skeleton serves as a mechanical support and reinforces the hybrid sponge to keep its shape during cell culture and transplantation. The cup-type hybrid porous scaffolds have another effect that the surrounding outside PLLA-collagen sponge cup can protect against cell leakage from the scaffold during cell seeding. The high cell seeding efficiency helps the scaffolds to hold more cells and decreases cell loss. The high porosity provides more space for cell accommodation and tissue formation. The hybrid scaffolds can be used for tissue engineering of skin [38,44], cartilage [39,40], bone [41–43] and ligament [37].

1.6 CONCLUSIONS

Many methods have been developed to control the pore structures and mechanical properties of polymeric porous scaffolds to meet the requirements for functional tissue engineering. Method using ice particulates as a template or porogen material can prepare collagen sponges with open surface pores, highly interconnected bulk pores and micropatterned structures. Hybridization of collagen sponges with biodegradable synthetic polymers can increase the mechanical property of collagen sponges.

All these porous scaffolds have precisely controlled pore and hybrid structures with unique property. They are very useful for tissue engineering of a variety of tissues and organs.

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