Tissue Engineered Scaffolds for Stem Cells and Regenerative Medicine

Hossein Hosseinkhani and Mohsen Hosseinkhani

Abstract For successful tissue regeneration, the cells constituting the tissue to be regenerated, such as matured, progenitor, or precursor cells, are necessary. Considering the proliferation activity and differentiation potential of cells, stem cells are practically promising. Among them, mesenchymal stem cells (MSCs) have been widely investigated for use by themselves or combined with scaffolds if necessary for promotion of cell proliferation and differentiation. It was found that MSCs have an inherent nature to differentiate into not only osteogenic linage cells but also chondrogenic, myogenic, adipogenic, and neurogenic lineages. MSCs have been experimentally used to demonstrate their in vivo potential to induce the regeneration of mesenchymal tissues. Since it is reported that the cells are effective in inducing the regeneration of tissues other than mesenchym, their feasibility in the cell source for regenerative medicine is highly expected. They are practically isolated from patients themselves. Material design of scaffold for cell proliferation and differentiation is one of the key technologies for tissue engineering. Porous materials with various dimensional structures have been investigated for the cell scaffold because they have a larger surface for cell attachment and proliferation than two-dimensional materials and are preferable to assist the formation of three-dimentional cell constructs, which may resemble the structure and function of body tissues. In addition, the three-dimentional scaffold also plays an important role in the substrate for in vitro cell culture to increase the number of cells as high as clinically applicable. This chapter reviews the basic principles of tissue engineering and the recent developments of stem cells for their potential applications in regenerative medicine.

Keywords Tissue engineering; Scaffolds; Stem cells; Regenerative medicine

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Introduction

Stem cells are a self-renewing cell type that can be differentiated into other cells. Conventional in vitro models to study differentiation of stem cells use freshly isolated cells grown in two-dimensional (2D) cultures. Clinical trails using in vitro stem cell culture can be expected only when the differentiated stem cells mimic the tissue regeneration in vivo. Therefore, the design of an in vitro three-dimensional (3D) model that mimics the in vivo environment is needed to effectively study its use for regenerative medicine. Biodegradable scaffolds play an important role for tissue-engineered scaffolds for tissue regeneration. Tissue-engineered scaffolds have a significant effect on stem cells' proliferation and differentiation. The application of scaffolding materials together with stem cell technologies is believed to hold enormous potential for applications in tissue regeneration. This chapter emphasizes that tissue-engineered scaffolds represent a viable strategy for the development of certain engineered tissue replacements and tissue regeneration systems using stem cells.

The Source of Stem Cells and Their Therapeutic Application

There are many limitations and problems that remain from current therapies such as autografts, allografts, xenografts, or metal prosthesis for the replacement of tissue defects resulting from tumors, surgical resections, trauma, or aging. Therefore, using stem cells for tissue regeneration represents a new direction toward regenerative medicine. Bone, cartilage, tendon, muscle, fat, and marrow stroma are formed during embryologic development ***(1)***. Bone marrow contains stromal or mesenchymal stem cells (MSCs) that have the ability to differentiate into osteoblasts, adipocytes, chondrocytes, or myoblasts ***(2, 3)***. Figure 1 shows the main source of stem cells and its differentiation capacity. Embryonic stem (ES) cells (fetal or adult cells) obtained from any germ layers such as ectoderm (epidermal tissues and nerves), mesoderm (muscle, bone, and blood), or endoderm (liver, pancreas, gastrointestinal tract, lungs) can differentiate into any cell type ***(4)***. The ability of stem cells to differentiate and replace mature cells is fundamental for regenerative medicine ***(5)***. In recent years, a combination of materials engineering and stem cells technology has been used to study tissue regeneration and ultimately mimic the stem cell niche ***(6)***. The application of stem cells for regenerative medicine is one of the most attractive research areas in biomedical engineering. There are some research trials under way in replacement therapy using stem cells, such as in infarcted heart, diabetes, and Parkinson's disease ***(6)***. Figure 2 indicates the differentiation tree of MSCs toward tissue regeneration by use of MSCs, precursor cells, and blast cells. In the field of regenerative medicine, scientists apply the principles of cell biology and materials engineering to construct biological substitutes that will restore and maintain normal function in injured tissues ***(7)***. Researchers have been investigating the fabrication of functional living tissue, or tissue engineering, using cells seeded on highly porous synthetic biodegradable polymer scaffolds as a novel approach toward

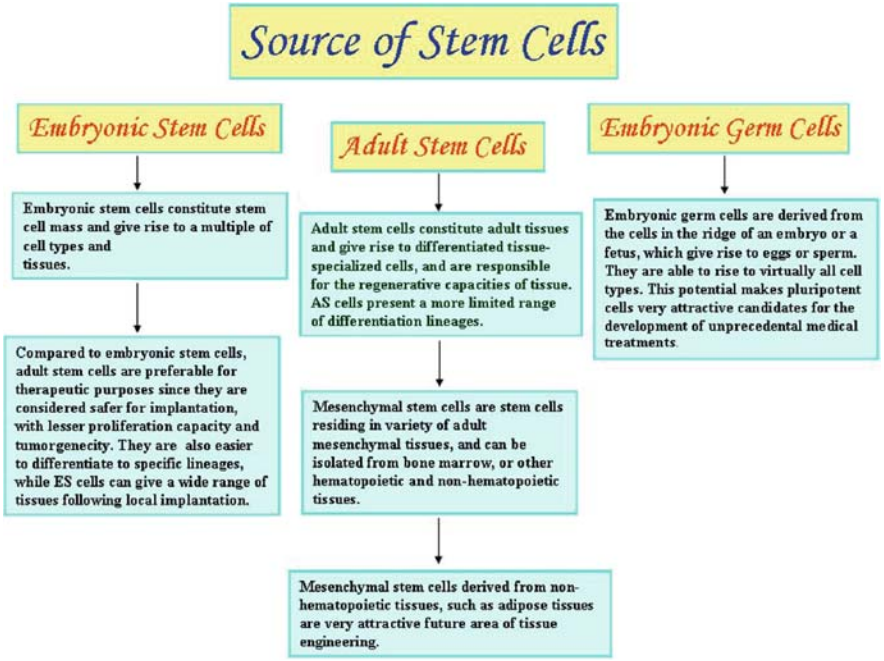

Fig. 1 The main source of stem cells

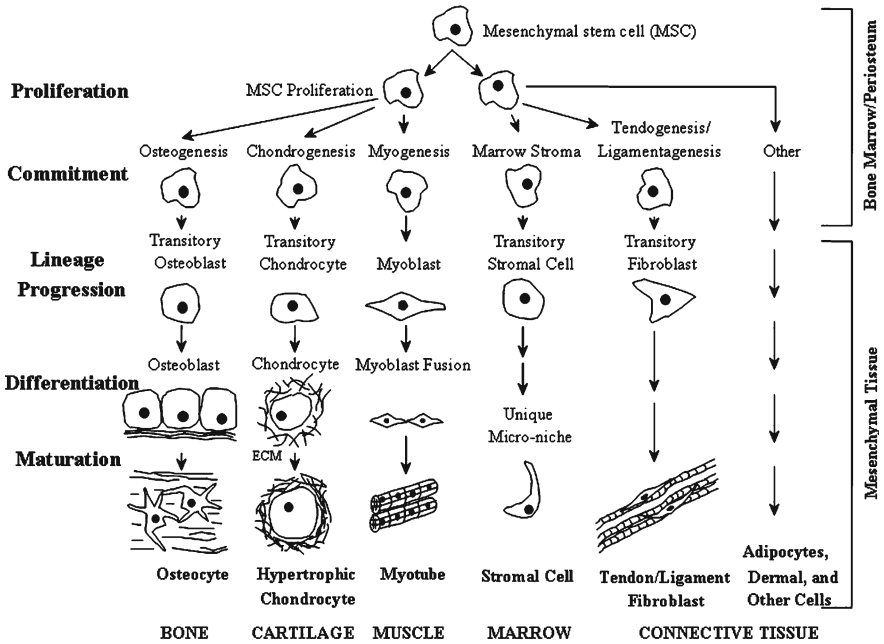

Fig. 2 Differentiation tree of stem cells

the development of biological substitutes that may replace lost tissue function ***(8)***. Over the past decade, scientists have applied the principles of tissue engineering in the fabrication of a wide variety of tissues, including both structural and complex tissues.

Tissue Engineering Principles in Stem Cells Technology

Regenerative medicine is a new field of science using stem cells to generate biological tissues and improve tissue functions. The application of MSCs has rapidly improved through research to evaluate their therapeutic applications ***(9)***. Tissue engineering is an interdisciplinary field that applies principles and methods of engineering toward the development of biological substitutes to improve the function of damaged tissue and organs ***(10, 11)***. The motivation of using tissue engineering in regenerative medicine centers around several factors:

1. Since 1970s, organ transplantation has become a common therapeutic approach for end-stage organ failure patients.
2. Demand is greater than supply; for example: 19,095 patients (1989), 80,766 patients (December 2002) on the UNOS National Patient Waiting List.
3. Cost of organ replacement therapy: $305 billion (US$, 2000).
4. The interdisciplinary approach of tissue engineering is a combinational technology, requiring the use of molecular biology, materials engineering, and reconstructive surgery.

For successful tissue regeneration, the cells constituting the tissue to be regenerated, such as matured, progenitor, or precursor cells, are necessary. Considering the proliferation activity and differentiation potential of cells, stem cells are most promising. Among them, MSC have been widely investigated for use by themselves or combined with scaffolds if necessary for promotion of cell proliferation and differentiation. It was found that MSC have an inherent nature to differentiate into not only osteogenic linage cells but also chondrogenic, myogenic, adipogenic, and neurogenic lineages ***(12–17)***. MSC have been experimentally used to demonstrate their in vivo potential to induce the regeneration of mesenchymal tissues ***(17–20)***. Since it is reported that the cells are effective in inducing the regeneration of tissues other than mesenchym, their feasibility in the cell source for regenerative medicine is highly expected. They are practically isolated from patients themselves ***(21–24)***. Material design of scaffold for cell proliferation and differentiation is one of the key technologies for tissue engineering. In conventional cell culture, such as static tissue culture dish (2D), the initial rate of cell growth is higher, but the proliferation stops once the cells have reached confluence. Porous materials with various dimensional (3D) structures have been investigated for the cell scaffold because they have a larger surface for cell attachment and proliferation than 2D materials and are preferable to assist the formation of 3D cell constructs, which may resemble the structure and function of body tissues. In addition, the 3D scaffold also plays an important

role in the substrate for in vitro cell culture to increase the number of cells as high as clinical application. Three-dimensional scaffolds, through their ability to regenerate or restore tissue and/or organs, have begun to revolutionize medicine and biomedical science. Scaffolds have been used to support and promote the regeneration of tissues. Different processing techniques have been developed to design and fabricate 3D scaffolds for tissue engineering implants. However, there is neither a simple nor an inexpensive method for producing the main characteristics that a scaffold should have for application in tissue engineering.

Because the proliferation of cells in the 3D scaffold needs oxygen and nutrition supply, the 3D scaffold materials should provide such an environment. Diffusion of nutrients, bioactive factors, and oxygen through 3D scaffolds is sufficient for survival of large numbers of cells for extended periods of time. A major constraint in the use of biodegradable polymer scaffolds for vascular tissue engineering is poor cell adhesion and lack of signals for new tissue generation. The presence of extracellular matrix (ECM) within the scaffold is desirable for growth of stem cells and in vitro formation of remodeled vascular conduit ***(25)***.

Tissue engineering is designed to regenerate natural tissues or to create biological substitutes for defective or lost organs by making use of cells. Considering the usage of cells in the body, it is no doubt that a sufficient supply of nutrients and oxygen to the transplanted cells is vital for their survival and functional maintenance ***(26)***. Without a sufficient supply, only a small number of cells that have been preseeded in the scaffold or migrated into the scaffold from the surrounding tissue would survive. Rapid formation of a vascular network at the transplanted site of cells must be a promising way to provide cells with the vital supply. This process of generating new microvasculature, termed neovascularization, is a process observed physiologically in development and wound healing ***(27)***. It is recognized that basic fibroblast growth factor (bFGF) functions to promote such an angiogenesis process ***(27, 28)***. The growth factor stimulates the appropriate cells (e.g., endothelial cells), already present in the body, to migrate from the surrounding tissue, proliferate, and finally differentiate into blood vessels ***(27)***. However, these proteins cannot always reach the sustained angiogenesis activity if they are only injected in solution form, most likely because of their rapid diffusional excretion from the injection site. One possible way for enhancing the in vivo efficacy is to achieve its controlled release over an extended time period by incorporating the growth factor in a polymer carrier. If this carrier is biodegraded or harmonized with tissue growth, it will work as a scaffold for tissue regeneration in addition to a carrier matrix for the growth factor release. The use of angiogenic factors is a popular approach to induce neovascularization. Among them, bFGF plays a multifunctional role in stimulation of cell growth and tissue repair. However, it has a very short half-life when injected and it is unstable in solution. To overcome these problems, bFGF was encapsulated within alginate, gelatin, agarose/heparin, collagen, and poly(ethylene-co-vinyl acetate) carriers ***(29–31)***. According to the results of these studies, it is conceivable to incorporate the angiogenic factor into a sustained releasing system and use it prior to the implantation.

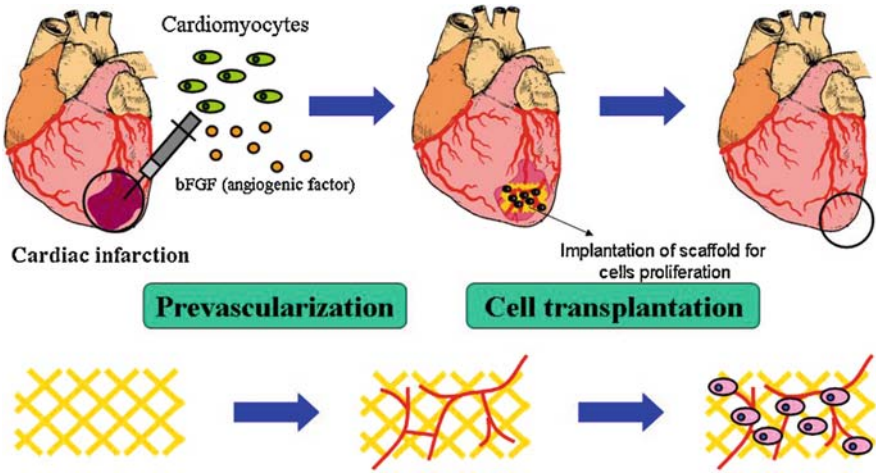

Fig. 3 Schematic illustration of tissue regeneration based on the principle of tissue engineering

Some studies have demonstrated that bFGF achieved promoted angiogenesis when used in combination with delivery matrices and scaffold ***(31–36)***. Figure 3 indicates tissue regeneration based on the principle of tissue engineering ***(37)***.

There are other growth factors currently used in tissue regeneration. Hepatocyte growth factor (HGF) was originally discovered as a protein factor to accelerate hepatocyte proliferation ***(38)***. Previous studies have demonstrated that HGF has great potential for proliferation, differentiation, mitogenesis, and morphogenesis of various cells ***(39–41)***. Therefore, HGF can be used for various tissue engineering applications where angiogenesis is needed. However, since growth factors such as HGF have a very short half-life, when they inject into the body, they lose their biological activities due to rapid digestion. Sustained-release technology has been used widely for different drugs and proteins to overcome this problem. Previous studies using growth factors such as bFGF, bone morphogenetic protein (BMP), and transforming growth factor (TGF) have demonstrated that their expected biological activities could be achieved when incorporated in carrier matrices ***(28, 42, 43)***. It has been shown that bFGF and vascular endothelial growth factor (VEGF) exhibited properties to promote the angiogenesis process ***(27, 31, 44)***. Osteogenic growth factors such as TGF-β, BMP, and bFGF can induce bone formation in both ectopic and orthotopic sites in vivo ***(45–47)***. Table 1 summarizes the characteristics of the growth factors used in tissue engineering.

Tissue-Engineered Scaffolds

The proposed technique of cell culture in 3D cell scaffold constructs is based on the use of 3D fibrous scaffold to guide cell organization. In comparison with conventional culture, cells maintained in 3D culture more closely resemble the in vivo environment with regard to cell shape and cellular environment that can influence the behavior of cells.

Table 1 Characteristics of the growth factors used in tissue engineering

Growth factor	Isoelectric point (IEP)	Molecular weight (kDa)	Biological substances for growth factor binding	Functions of growth factor
Basic fibroblast growth factor (bFGF)	9.6	16	Heparin or heparan sulfate	Stimulating the cells involved in the healing process (bone, cartilage, nerve, etc.). Angiogenesis
Transforming growth factor β1 (TGF-β1)	9.5	25	Heparin or heparan sulfate Collagen type IV Latency associated protein Latent TGF-β1 binding protein	Enhancing the wound healing, stimulating the osteoblast proliferation to enhance bone formation
Bone morphogenetic protein-2 (BMP-2)	8.5	32	Collagen type IV	Stimulating the mesenchymal stem cells to osteoblast lineage and inducing the bone formation both at bone and ectopic sites
Vascular endothelial growth factor (VEGF)	8.5	38	Heparin or heparan sulfate	Stimulating the endothelial cell growth, angiogenesis, and capillary permeability
Hepatocyte growth factor (HGF)	5.5	100	Heparin or heparan sulfate	Stimulating of matrix remodeling and epithelial regeneration (liver, spleen, kidney, etc.)

It has been recognized that induction of tissue regeneration based on tissue engineering can be achieved through three key steps: the proliferation of cells, the seeding of cells and proliferation in a suitable scaffold, and the maintenance of the differentiation phenotype of the engineered tissues ***(48)***. The property of scaffold material for cell attachment is one of the major factors contributing its morphology, proliferation, functions, and the subsequent tissue organization ***(49)***. At first, cells attach to the material surface of scaffold, then spread, and proliferate. The 3D scaffold can provide a larger surface area available for cell attachment and spreading than a 2D scaffold could (i.e., tissue culture plate). Xie et al. ***(50)*** have reported that the initial rate of cells growth was higher for the 2D culture, but once the cells reached confluence, their proliferation stopped. However, the cells' growth in the 3D scaffold was continued for longer time periods than that of the 2D scaffold. Other reports have demonstrated that cell proliferation was superior in the 3D scaffold versus the 2D scaffold ***(51–55)***.

Regenerative medicine is an interdisciplinary field that combines engineering and live sciences in order to develop techniques that enable the restoration, maintenance, or enhancement of living tissues and organs. Its fundamental aim is the creation of natural tissue with the ability to restore missing organ or tissue function, which the organism has not been able to regenerate in physiological conditions. As a result, it aspires to improve the health and quality of life for millions of people worldwide

and to provide a solution to the present limitations of rejections, low quantity of donors, and so forth ***(56)***. Tissue engineering needs a scaffold to serve as a substrate for seeding cells and as a physical support in order to guide the formation of the new tissue ***(56–61)***. The majority of researchers use techniques that utilize 3D polymeric scaffolds that are composed of natural or synthetic polymers. Synthetic materials are attractive because their chemical and physical properties (e.g., porosity, mechanical strength) can be specifically optimized for a particular application. The polymeric scaffolds structures are endowed with a complex internal architecture, channels, and porosity that provide sites for cell attachment and maintenance of differentiated function without hindering proliferation ***(56)***. Ideally, a polymeric scaffold for tissue engineering should have the following characteristics: (a) appropriate surface properties promoting cell adhesion, proliferation, and differentiation, (b) biocompatibility, (c) highly porous, with a high surface area to volume ratio, with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste, and (d) mechanical properties sufficient to withstand any in vivo stresses ***(56, 57, 61–65)***. The last requirement is difficult to combine with the high porosity in a large volume of material. That is why it is necessary to use polymeric matrices with special or reinforced properties, especially if the polymer is a hydrogel.

Among many materials currently used as cell scaffolds, collagen has been the most widely used. Its in vivo safety has been proven through the long-term applications in clinical medicine, cosmetics, and foods. The collagen sponge fabricated by freeze-drying method, followed by crosslinking of combined dehydrothermal, glutaraldehyde, and ultraviolet (UV), is highly porous with an interconnected pore structure. This method is effective in the infiltration of cells and for supplying oxygen and nutrients to the cells or excluding the cells wastes, while the shape and bioresorbability can be readily regulated by changing the formulation conditions. However, as shown in Fig. 4, the drawback of using a collagen sponge as a scaffold for cell proliferation and differentiation is its poor mechanical strength. To overcome the inherent material problem of the sponge, the combination with other materials has been attempted. Considering implantation, the materials to be combined should also be bioabsorbable. From the viewpoint of clinical application, it is preferable to select the material that has been clinically used. Several biodegradable synthetic polymers, such as poly(glycolic acid) (PGA) and its copolymers with L-lactic acid, DL-lactic acid, and β-caprolactone, have been fabricated into the cell scaffolds of nonwoven fabric and sponge shapes for tissue engineering. The mechanical resistance of the scaffolds to compression is practically acceptable for the tissue engineering applications because of their hydrophobic nature. However, the cell attachment to the surface of synthetic polymer scaffolds is poor compared with that of collagen. PGA has been approved by the U.S. Food and Drug Administration for clinical applications. Our previous study revealed that incorporation of PGA fiber enabled a collagen sponge to increase the resistance to compression in vitro and in vivo ***(66)***. The in vitro culture experiment revealed that the number of MSC attached increased with the incorporation of PGA fiber to a significantly higher extent compared with that of the original collagen sponge ***(66)***. It is key for the present technology to fabricate mechanically strong collagen sponges

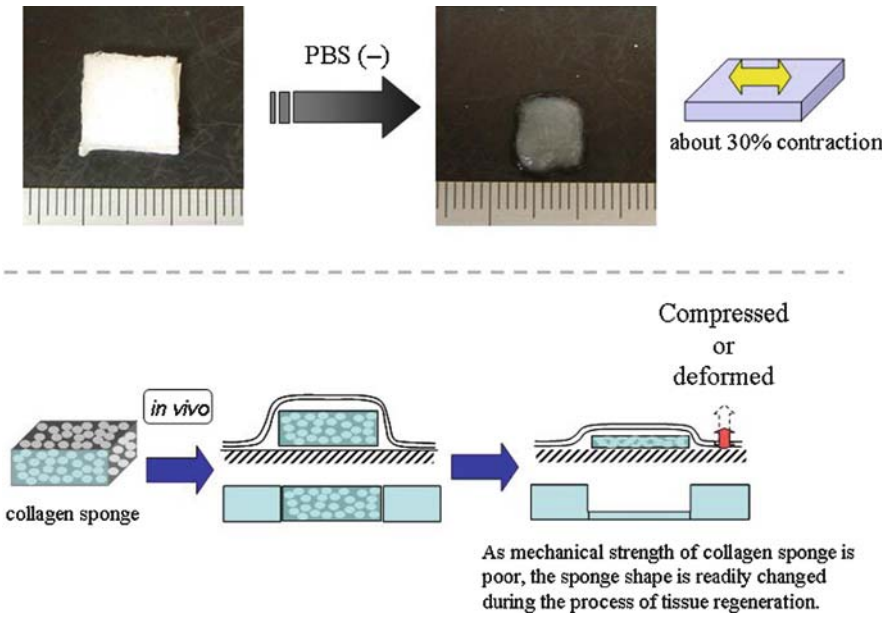

Fig. 4 Contraction of collagen sponge by in the presence of biological substances

by incorporating the PGA fiber of which the amount is as low as possible. Since collagen is more compatible to cells than PGA, at a higher amount of PGA fiber incorporated, the fiber may cause inflammation response to the sponge. Moreover, the collagen sponge does not become strong enough to resist the compressed deformation only by increasing the extent of crosslinking. Because the PGA fiber incorporation also suppressed the shrinkage of collagen sponge, it is possible that the volume available for cell attachment was larger, resulting in a higher number of cells attached. We have shown that mouse fibroblast L929 cells infiltrated into the collagen sponge incorporated PGA fiber more deeply than the collagen sponge alone (Figs. 5 and 6). This phenomenon also can be explained in terms of suppressed shrinkage of sponge by PGA fiber incorporation. The collagen sponge mechanically reinforced by PGA fiber incorporation is a promising scaffold for tissue regeneration. The incorporation of PGA fiber enabled the sponge to increase the resistance to compression. In comparing in vivo degradability, the collagen scaffold is generally digested faster than the PGA fabric. This degradation profile greatly depends on the crosslinking extent of collagen sponge and the molecular weight of PGA and the formulation shape. In our study, a combined crosslinking method of dehydrothermal, glutaraldehyde, and UV was used to prepare collagen sponges with or without PGA fiber incorporation. Weadock et al. ***(67)*** have evaluated the physical, mechanical, and biological behaviors of collagen sponge crosslinked by physical (UV irradiation and dehydrothermal) and chemical (carbodiimide and glutaraldehyde) or a combination of physical (dehydrothermal) and chemical (carbodiimide) crosslinking.

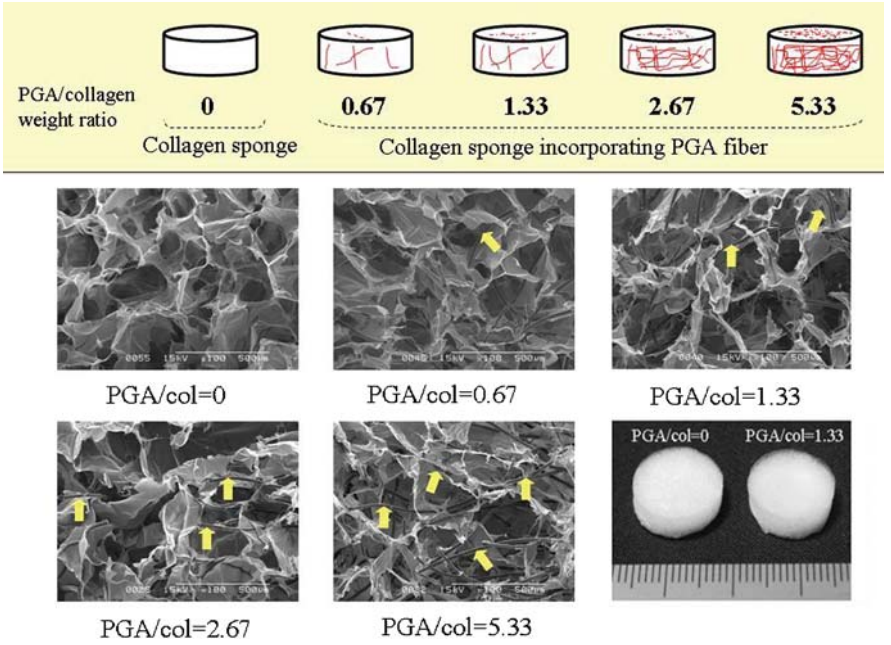

Fig. 5 Cross-sectional scanning electron microscopy (SEM) photographs of the structural morphology of collagen sponge with different poly(glycolic acid) (PGA) fiber incorporation. *Arrows* shows the location of PGA fibers inside the collagen sponge. The *right panel* in bottom *brown color* shows light microscopic photographs of a collagen sponge without PGA fiber incorporation and a collagen sponge incorporating PGA fiber

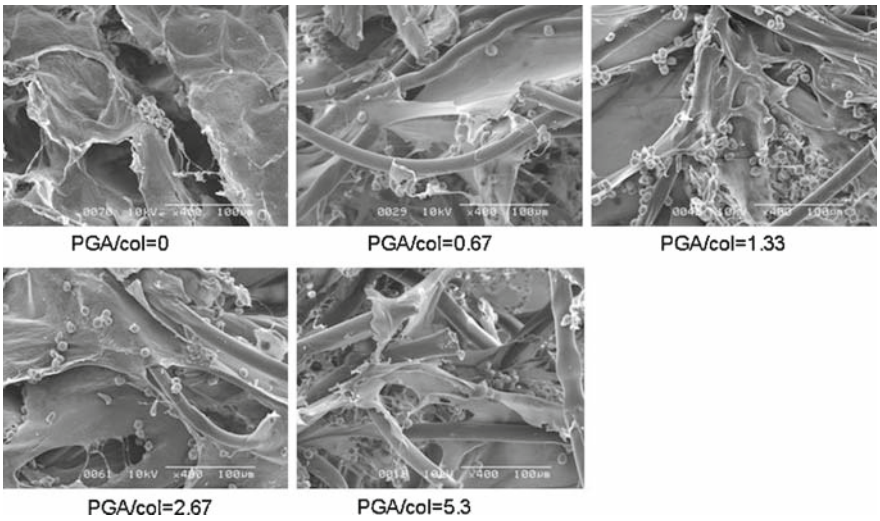

Fig. 6 Cross-sectional scanning electron microscopy (SEM) photographs of the structural morphology of collagen sponge with different poly(glycolic acid) (PGA) fiber incorporation 2 days after culturing the mouse fibroblast L929 cells

The results revealed that combination of physical (dehydrothermal) and chemical (carbodiimide) crosslinking of collagen reduced significantly the swelling ratio and increased the collagenase resistance time and low- and high-strain modulus compared with a single crosslinking of UV, dehydrothermal, and carbodiimide. The glutaraldehyde crosslinking itself showed the same physical and mechanical properties as the combination of physical (dehydrothermal) and chemical (carbodiimide) crosslinking.

The polymeric scaffold design depends on its anticipated application, but in any application it must achieve a structure with the aforementioned characteristics, which are necessaries to their correct function. Successfully achieving this is conditional on two factors: the materials used, both the porogen, and the reticulate polymer, which is infiltrated in the porogen to become a scaffold; and the structural architecture, both external and internal, basically shown by its porosity (high surface area to volume ratio), geometry, and pore size, keeping in mind that the structures must be easily processed into three-dimensional format. On the basis of the extensive range of polymeric materials, different processing techniques have been developed to design and fabricate 3D scaffolds for tissue engineering implants ***(56, 57, 61, 68–74)***. They include (a) phase separation, (b) gas foaming, (c) fiber bonding, (d) photolithography, (e) solid free form (SFF), and (f) solvent casting in combination with particle leaching.

However, none of the techniques have achieved a suitable model of 3D architecture so that the scaffolds can fulfill their purpose in the desired way using high-cost equipment, for the reasons discussed below. When using phase separation, a porous structure can be easily obtained by adjusting thermodynamic and kinetic parameters. However, because of the complexity of the processing variables involved in the phase-separation technique, the pore structure cannot be easily controlled. Moreover, it is difficult to obtain large pores, which may exhibit a lack of interconnectivity ***(57, 58, 71)***. Gas foaming has the advantage of room temperature processing but produces a largely nonporous outer skin layer and a mixture of open and closed pores within the center, leaving incomplete interconnectivity. The main disadvantage of the gas foaming method is that it often results in a nonconnected cellular structure within the scaffold ***(58, 71)***. Fiber bonding provides a large surface area for cell attachment and a rapid diffusion of nutrients in favor of cell survival and growth. However, these scaffolds, as the ones used to construct a network of bonded PGA, lacked the structural stability necessary for in vivo use. In addition, the technique does not lend itself to easy and independent control of porosity and pore size ***(56–58)***. Photolithography has also been employed for patterning and obtaining structures with high resolution, although this resolution may be unnecessary for many applications of patterning in cell biology. In any case, the disadvantage of this technique is the high cost of the equipment needed, which limits its applicability ***(75)***. SFF scaffold manufacturing methods provide excellent control over scaffold external shape, internal pore interconnectivity, and geometry, but it offers limited microscale resolution. Also, it is important to consider the following items: (1) the minimum size of global pores is 100 μm, (2) SFF requires complex correction of scaffold design for anisotropic shrinkage during fabrication; and (3) it requires high-cost equipment ***(63)***.

Finally, solvent casting in combination with particulate leaching method, which involves the casting of a mixture of monomers and initiator solution and a porogen in a mold, termed polymerization, followed by leaching-out of the porogen with the proper solvent to generate the pores, is inexpensive but still has to overcome some disadvantages in order to find engineering applications, namely the problem of residual porogen remains, irregular shaped pores, and insufficient interconnectivity ***(61, 76)***. The proposed scaffolds may find application as structures that facilitate either tissue regeneration or repair during reconstructive operations ***(56, 77, 78)***. The new structure could also find application in other areas in which the pore morphology might play an essential role, such as membranes ***(79)*** and filters ***(80)***. In the United States alone, each year over 10,000 newly injured people are added to the total of more than 250,000 who are confined to a wheelchair ***(81)***. A major limitation in treating nerve injury, central nervous system (CNS), and peripheral nervous system (PNS) is the failure of current therapies to induce nerve regeneration. Unfortunately, for CNS injury, and particularly spinal cord injury, there is currently no treatment available to restore nerve function ***(82)***. One possible avenue to remedy this situation is to artificially engineer nerve tissue. It is commonly accepted that physical guidance of axons is a vital component of nerve repair. Many materials have been used in an attempt to physically guide the regeneration of damaged nerves ***(82)***. Kang et al. ***(83)*** have concluded that preferential alignment of channel pores may provide a unique advantage in certain medical applications, such as nerve regeneration. In another research work, Blacher et al. ***(78)*** fabricated a highly oriented poly-lactic acid (PLA) scaffold for spinal cord regeneration and demonstrated that highly oriented macroporous have efficiency in axonal regeneration both in the PNS and CNS. Cell migration and angiogenesis were observed, as well as the expected orientation of axonal growth. The axons were perfectly aligned along the pore direction, which confirmed the crucial role of 3D polymer structures. Plant et al. ***(84)*** have demonstrated that 3D sponges of poly-hydroxy ethyl methacrylate (PHEMA) sponges are able to house a purified population of glial cells and provide a scaffold for regenerative growth of axons in the lesioned rat optic tract and may be a candidate for use as prosthetic bridges in the repair of the damaged CNS. However, they deduce that further work is necessary to optimize their procedure, such as providing a more oriented trabecular network within the hydrogel scaffold. In the research carried out by Shugens et al. ***(85)***, macroporous foams of 100 μm were produced in the form of channels by the solid–liquid phase separation technique for nerve regeneration. They concluded that nerve regeneration can only occur through a structure of interconnected pores of ideal diameter in the range of 10–100 μm. In the study developed by Maquet et al. ***(86)***, poly(D,L-lactide) foams with macroporous of 100 μm organized longitudinally were prepared by freeze-drying technique for spinal cord regeneration. They showed that the parallel assembly of rods of porous (diameter ~100 μm) containing an amphophilic copolymer was a promising strategy to bridge a defect in the spinal cord of adult rats, and they confirmed a high density of cells in the surface of porous interconnected structures as well.

Porosity of fabricated scaffolds can be determined through the measurement of the apparent density of the scaffold. For this, distilled water is used as a filler of the porous structure. The dried scaffold is weighted and placed in a glass tube connected to a vacuum pump then filled with distilled water before breaking the vacuum. The scaffolds filled with water are weighed again and the porosity is calculated as:

$$\Pi(\%) = \frac{V_p}{V_t} \times 100 = \frac{m_l / \rho_l}{\frac{m_l}{\rho_l} + \frac{m_m}{\rho_m}} \times 100,$$

where Π is the porosity, V_p and V_t are the volume occupied by the pores and the volume of the scaffolds, respectively, ρ_l is the density of the filler liquid and ρ_m is the bulk density of the scaffolding material, m_l and are m_m the liquid mass and dried scaffold mass, respectively.

Tissue Engineered Nanoscaffolds

The design of materials that can regulate cell behavior, such as proliferation and differentiation, is a key component for the fabrication of tissue engineering scaffolds. From the viewpoint of immune system response of the body, the implanted biomaterials should mimic the structure and biological function of native ECM, both in terms of chemical composition and physical structure as reported by Ma et al. ***(87)***. Therefore, in order to mimic the biological function of ECM proteins, the scaffold materials used in tissue engineering need to be chemically functionalized to promote tissue regeneration as ECM does. It has been reported that collagen and elastin as ECM proteins are made from fibers in dimensions smaller than micrometers ***(87)***. It seems that artificial nanoscaled fibers have great potential application in the field of biomaterials and tissue engineering.

The initial report showed that nanoscaled features influenced cell behaviors ***(88)***. Nanoscaled surface topography has been found to promote osteoblast adhesions ***(89)***. It has been demonstrated that osteoblast adhesion, proliferation, alkaline phosphatase activity, and ECM secretion on carbon nanofibers increased with decreasing fiber diameter in the range of 60–200 nm, whereas the adhesion of other kinds of cells such as chondrocytes, fibroblasts, and smooth muscle cells was not influenced ***(90, 91)***. It has been supposed that the nanoscaled surface affects the conformation of adsorbed adhesion proteins such as vitronectin, thus affecting the cell behaviors ***(92)***. In addition, the nanoscaled dimensions of cell membrane receptors such as integrins should also be considered. It has been reported that there are three different approaches toward the formation of nanofibrous materials: phase separation, electrospinning, and self-assembly ***(93)***. Phase separation and self-assembling of biomolecules can generate smaller diameter nanofibers in the same range of natural ECM, while electrospinning generates large diameter nanofibers

on the upper end of the range of natural ECM ***(93)***. Electrospinning is a common technique used to fabricate tissue engineering scaffolds ***(94)***. It is an easy technique and extremely inexpensive and can be applied for many different types of polymers. The authors' recent study demonstrated that fabricated PGA/collagen nanofibers through electrospinning significantly enhanced cell adhesion compared with PGA/collagen microfibers ***(95)***. Figure 7 shows a schematic illustration of an electrospinning device for the fabrication of nanofibers, while Fig. 8 shows cross-sectional scanning electron microscopy (SEM) photographs of the structural morphology of PGA/collagen nanofibers fabricated by electrospinning before and after culturing the MSC.

One of the most common approaches to produce fibers similar to ECM proteins such as collagen is self-assembly. It has been shown that peptide amphiphile (PA) contains a carbon alkyl tail and several other functional peptide formed nanofibers through self-assembly by mixing cell suspensions in media with dilute aqueous solutions of the peptide ***(96)***. Another type of peptide containing 16 alternating hydrophobic and hydrophilic amino acids was fabricated to self-assemble into nanofibers under appropriate pH values ***(97)***. Nanoscaled fibers produced by self-assembly of PA may be a promising approach in designing the next generation of biomaterials for drug delivery and tissue engineering.

It would be beneficial for biomedical applications if scaffold materials could promote the adhesion and growth of cells on their surfaces. The sequence of arginine–glycine–aspartic acid (RGD) has been discovered as a cell attachment sequence in various adhesive proteins present in the ECM and found in many proteins, such as fibronectin, collagen type 1, vitronectin, fibrin, and von Willebrand factor ***(98)***. It has been well recognized that the sequence of RGD interacts with various types of integrin receptors of mammalian cells. Since the discovery of the

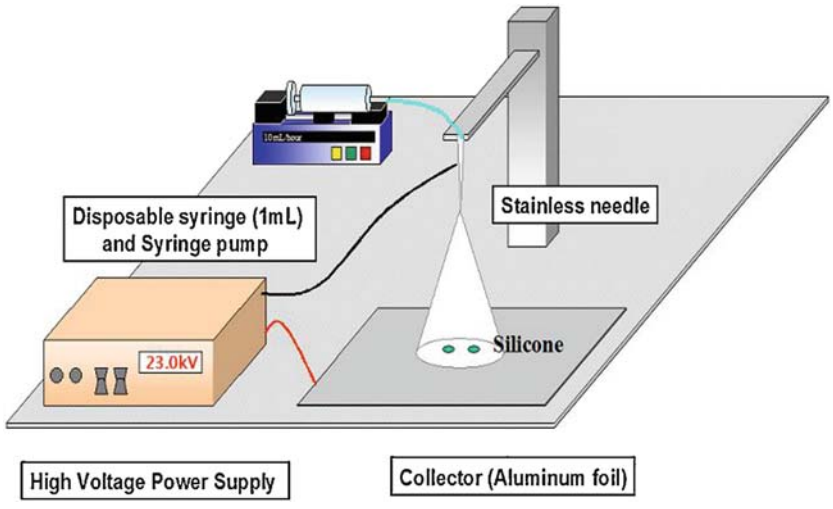

Fig. 7 Schematic illustration of electrospinning machine

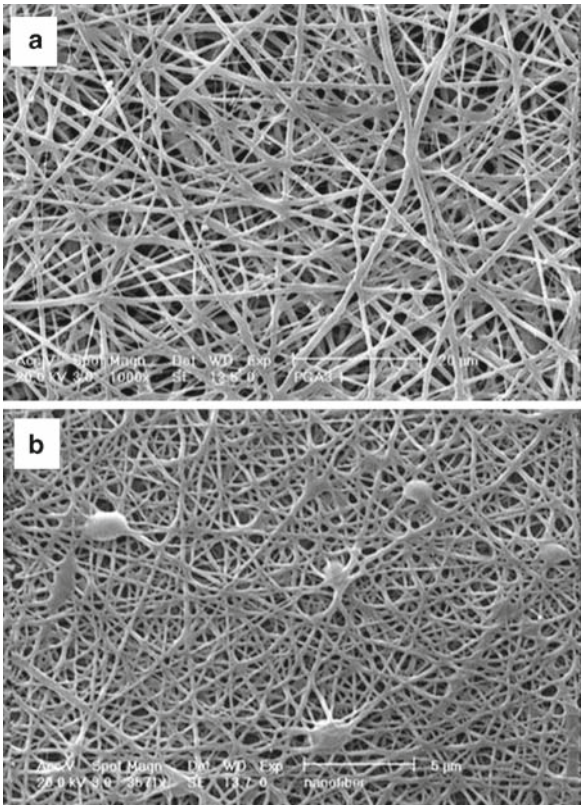

Fig. 8 Cross-sectional scanning electron microscopy (SEM) photographs of the structural morphology of poly(glycolic acid) (PGA)/collagen nanofibers fabricated by (**a**) electrospinning and (**b**) 2 days after culturing the mesenchymal stem cells (MSC) on nanofibers

RGD sequence as a cell attachment sequence in adhesive proteins of the ECM, there have been several efforts to synthesize bioactive peptides incorporating RGD for therapeutic purpose ***(99)***. Micro- and nanopatterned scaffolds have been less well investigated in regard to stem cells, although two recent studies highlight their attractiveness ***(100)***. In one study, Silva et al. ***(101)*** included a five amino acid, laminin-specific, cell-binding domain (which binds to specific integrins on cell surface) at the hydrophilic head of their amphiphiles and showed that neural stem cells could be induced to differentiate into neurons when cultured within a peptide gel. In contrast, cells grown in control scaffolds without the laminin-specific domain or on 2D tissue culture plastic coated with laminin solution differentiated much less. This was hypothesized to be largely a result of the density of the cells' binding ligands to which the cells were exposed, indicating clearly the importance of ECM in influencing cell function. Our recent studies have indicated that when the laminin-specific domain in the amphiphilic molecule was replaced with the amino acid sequence, RGD, a common cell-binding domain in many ECM proteins,

especially collagen, differentiation of MSC to osteoblasts was significantly enhanced compared with amphiphilic nanofibers without this sequence or to 2D controls ***(105)***. This is because the interaction of MSC integrins receptors with RGD of the peptide enhanced cell attachment on peptide nanofibers. The artificial scaffolds formed by self-assembling molecules not only provides a suitable support for cell proliferation but also serves as a medium through which diffusion of soluble factors and migration of cells can occur. The result of the cell attachment and proliferation revealed that diffusion of nutrients, bioactive factors, and oxygen through these highly hydrated networks is sufficient for survival of large numbers of cells for extended periods of time.

As understood from the findings, proteins and peptides can self-assemble into various structures like nanotubes, nanovesicles, and 3D peptide matrices with interwoven nanofibers. Macroscopic 3D peptide matrices can be engineered to form various shapes by changing the peptide sequence. Self-assembled peptide materials encouraged cell proliferation and differentiation. In regenerative medicine, these peptide matrices were used to cultivate chondrocyte ECM that can be used to repair cartilage tissue. Thus cartilage tissue engineering has been done by placing the primary chondrocytes and MSCs into these self-assembled peptide hydrogels to produce collagen and glycosaminoglycans. These peptide matrices can also be used in regeneration of bone by incorporating a phosphorylated serine that can attract and organize calcium ions to form hydroxyapatite crystals and functionalize them with a cell adhesion motif like RGD acid complex. The research studies have not been limited only to natural amphiphilic peptides. There are many research trials that have focused on synthesizing complex amphiphilic peptides by joining hydrophilic peptides into long alkyl chains. The peptide end of the molecule was designed to function and regulate biomineralization. Bone is produced as a result of deposition of calcium and phosphate ions to form hydroxyapatite crystals. This process is known as mineralization. Serine is a nonessential amino acid. When a phosphorylated serine was incorporated with the synthetic amphiphilic peptide complex, it served to attract and organize calcium and phosphate ions to form hydroxyapatite crystals. Furthermore, the synthetic amphiphilic peptides have been functionalized by adding a cell-adhesion motif. It was the RGD that was attached to the C-terminus of the peptide. This can be used to study the ability of bone cells to differentiate, proliferate, and adhere to a biomaterial surface like titanium. Titanium is the most widely used biomaterial surface to produce orthopedic implants, dental implants, and hip replacements. In spite of its excellent biocompatibility, titanium implants still fail. Most orthopedic implants have a lifetime of 15 years to the maximum. In order to produce a newer version of titanium implants that can stay in the body for a longer period of time, its surface has to be modified with nano-sized surface patterns so that bone cells (osteoblasts) differentiate and migrate into these patterns for better bone-implant adhesion. For such a purpose, these synthetic amphiphiles can be used to regulate and control the osteoblasts ***(71, 102)***.

Our recent study has indicated that a 3D networks of self-assembled nanofibers was formed by mixing a bFGF suspension with aqueous solution of PA as an injectable carrier for controlled release of growth factors and was then used for feasibility of

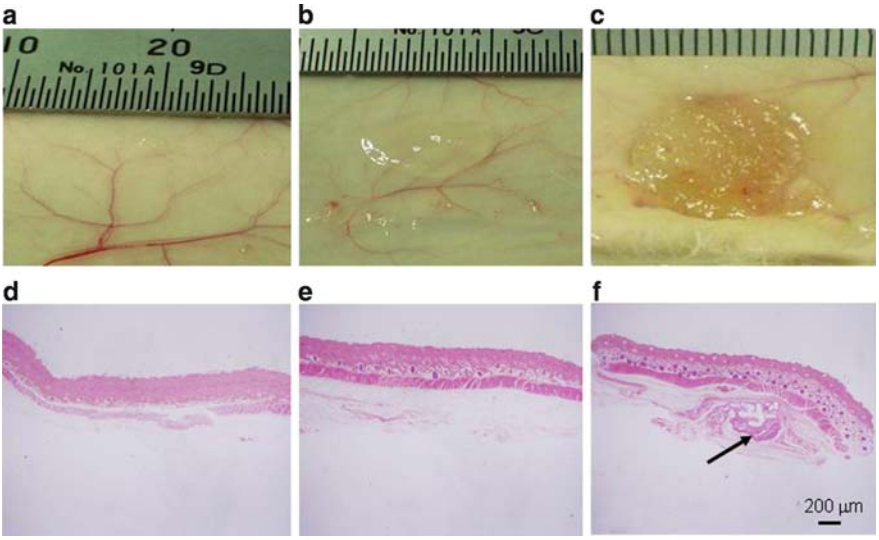

Fig. 9 Representative of tissue appearance (**a–c**) and histological cross-sections (**d–f**) of ectopically formed bone after subcutaneous injection of (**a**, **d**) transforming growth factor β (TGF-β), (**b**, **e**) peptide solution, and (**c**, **f**) peptide solution with TGF-β. The concentration of TGF-β is 10 µg. Each specimen subjected to hematoxylin and eosin staining. *Arrow* indicates the newly formed bone

prevascularization by the bFGF release from the 3D networks of nanofibers in improving efficiency of tissue regeneration ***(103)***. The bFGF incorporated in alginate, gelatin, agarose/heparin, collagen, and poly(ethylene-co-vinyl acetate) releasing system ***(28–31)*** requires surgery for implantation, which is not always a welcomed option. In contrast, the bFGF incorporated in self-assembled peptide could be delivered to living tissues by simply injecting a liquid (i.e., PA solutions) and bFGF solution. The injected solutions would form a solid scaffold at the injected site of tissue and the release bFGF would induce significant angiogenesis around the injected site, in marked contrast to bFGF injection alone or PA injection alone ***(102)***. This release system also was able to induce significant bone formation when PA solutions and TGF-β were subcutaneously injected to the back of rat (Fig. 9). The injected solutions of peptide and TGF-β formed a solid gel, and the sustained release of TGF-β induced significant ectopic bone compared with TGF-β injection. As a flexible delivery system, these scaffolds can be adapted for sustained release of many different growth factors and biomolecules.

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