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Effects of Three-dimensional Scaffolds on Cell Organization and Tissue Development

Yan Li and Shang-Tian Yang*

Department of Chemical Engineering, The Ohio State University, Columbus, OH 43210, USA

Abstract Tissue engineering scaffolds play a critical role in regulating the reconstructed human tissue development. Various types of scaffolds have been developed in recent years, including fibrous matrix and foam-like scaffolds. The design of scaffold materials has been investigated extensively. However, the design of physical structure of the scaffold, especially fibrous matrices, has not received much attention. This paper compares the different characteristics of fibrous and foam-like scaffolds, and reviews regulatory roles of important scaffold properties, including surface geometry, scaffold configuration, pore structure, mechanical property and bioactivity. Tissue regeneration, cell organization, proliferation and differentiation under different microstructures were evaluated. The importance of proper scaffold selection and design is further discussed with the examples of bone tissue engineering and stem cell tissue engineering. This review addresses the importance of scaffold microstructure and provides insights in designing appropriate scaffold structure for different applications of tissue engineering.

Keywords: tissue development, fibrous matrix, foam-like scaffold, microstructure

INTRODUCTION

Tissue engineering combines biological knowledge and engineering principles toward the goal of constructing the substitutes of various tissues and organs. It generally utilizes a natural or synthetic matrix as the physical scaffold to provide three-dimensional (3-D) guidance [1,2]. The properties of tissue scaffolds determine the development of engineered tissues or organs because the 3-D scaffold is in direct contact with human cells and it provides physical cues to regulate cell proliferation and differentiation. Fig. 1 illustrates the basic relationship between the scaffold and the series of cellular events that determine the tissue development. Therefore, the design of appropriate matrix materials with the desired structure is an essential issue in order to regulate the cell-cell and cell–matrix interactions. It is important to get cells to grow and to stop growing, to differentiate and to stop differentiating by controlling the extracellular environment. The 3-D scaffold is one of the most important components in this environment.

In vivo-like culture conditions are preferred in all tissue engineering studies. 3-D scaffolds can be fabricated into the desired structure and shape as the template to facilitate the tissue regeneration for various types of tissues or organs. The biodegradable polymer PLA foam was fabricated into complex geometries such as in the shape of a human nose to guide tissue generation [3] PGA nonwoven matrix was configured into the bladder shape and the functional bladder was reconstructed from urothelial and smooth muscle cells [4]. A functional artery was constructed from a PGA scaffold with tubular structure [5]. A morphological and functional equivalent of the human cornea was developed *in vitro* from three layers of stromal, epithelial and endothelial cells on a collagen-chondroitin sulfate substrate to simulate *in vivo* cornea constitution [6]. To meet the requirements of various tissue engineering applications, a variety of tissue engineering scaffolds have been developed in recent years, including PLGA foam, collagen gel, alginate sponge, hydrogel, and fibrous matrix. The chemistry, biodegradability, mechanical strength, and physical structure of the scaffold have potent regulating effects on tissue development. Cell-matrix interactions mainly involve cell-surface interactions and cell-structure interactions. The earlier studies of 3-D scaffolds focused on cell-surface interactions; however, the importance of the microstructure of 3-D scaffolds in guiding tissue regeneration was also realized in recent years. In this review, the effect of surface properties will be briefly summarized first to address the importance of cell-surface interactions. Since the effect of 3-D scaffold microstructure has not been sufficiently reviewed so far, especially for fibrous matrices, the effects of the microstructure of 3-D scaffolds including foam, sponge, and fibrous matrix for various tissue engineering applications will be discussed in details. In addition, the influence of other properties such as mechanical strength and dimensions will be mentioned briefly.

* **Corresponding author**
Tel: +1-614-292-6611 Fax: +1-614-292-3769
e-mail: yang.15@osu.edu

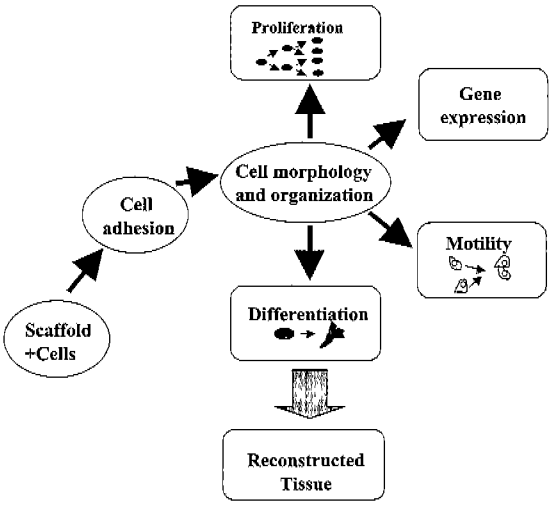

Fig. 1. Interactions between tissue scaffolds and cultured cells in their morphology, spatial organization, proliferation, differentiation, and function.

Table 1. Surface properties affecting cell-surface interactions

Property	Material	Application	Reference
Surface chemistry	ECM concentration	Capillary	[9]
	Different materials (PGA, PLGA, Collagen)	Smooth muscle	[11]
Surface geometry	Micro-pattern	Capillary endothelial cells	[21,22]
	Microgroove	Hepatocyte, fibroblast	[24,25, 26]
	Surface pore (PTFE porous membrane)	Neovascularization	[54]
	Surface curvature	*In vivo* tissue response	[48]
Surface biological activity	RGD binding	Osteoblast-like cells	[27]
		Nerve, endothelial cells	[29]
	Growth factor incorporation (*e.g.* EGF)	Epidermal cells	[31]

CELL-SURFACE INTERACTIONS

In 3-D tissue culture, cell-surface interaction is an important process for cell adhesion and subsequent tissue development. The insight of cell-surface interaction in 3-D scaffolds usually is obtained from cultures on 2-D substrate. Surface chemistry, surface geometry, and surface biological activity of 3-D scaffolds take active roles in regulating human tissue development. Table 1 summarizes the effects of various surface properties on different engineered tissues. The details are discussed below.

Effects of Surface Chemistry

Surface chemistry is defined as the chemistry of the outermost functional groups of a surface (nm scale). The difference in surface chemistry could be sensed by certain types of cells. For example, chondrocytes can discriminate between Ti and TiO_2 with different expression levels of cellular alkaline phosphatase activity [7]. Cell proliferation and differentiation are usually regulated through ECM proteins adhering on surface. If the surface was directly coated using ECM protein solution (*e.g.* fibronectin or laminin) with different concentrations, cell shape can be controlled by adjusting ECM density [8,9]. Spreading cells were found at high density and round cells at low density, thus cell growth (spreading cells) and differentiation (round cells) were regulated correspondingly. Different surface chemistries induced different amounts or patterns of ECM protein adsorption. For example, the adsorption of poly-L-lysine and collagen were more effective for PLA compared to PGLA [10]. Vitronectin was the major adsorbed protein when smooth muscle cells were cultured on PGA scaffolds while both fibronectin and vitronectin were detected on type I collagen sponge [11]. As a result, the phenotypes of smooth muscle cells, indicated by elastin and collagen synthesis, were regulated by scaffold chemistry independent of physical form. The effect of surface chemistry was also recognized using surfaces with same groove patterns but different materials. The contact guidance was altered on different materials although the surface pattern was the same. Rat dermal fibroblasts strongly aligned to the surface grooves and had a more rounded morphology on PLA than on other materials including polystyrene and Ti-coated polystyrene. In addition, the cells contracted and deformed the surface groove when using silicone substrate [12]. Various physical or chemical modifications of the surface improved the ECM adsorption from the serum. Plasma discharge changed the contact angle, wettability and the composition of chemical elements, and induced functionalization of the surface [13]. Chemical reactions that occurred on surface, such as hydrolysis and glycolysis, also changed the contact angle and the ratio of hydrophobicity and hydrophilicity on polyester surfaces and improved cell adhesion [14].

The search continues for new materials in order to fabricate different 3-D scaffolds for various tissue engineering applications. New biomaterials such as chitosan [15], tyrosine-derived polycarbonates/polyarylates [16], hyaluronic acid benzyl ester [17], poly(anhydrides)/poly(orthoesters) [11], and alginate [18] have been investigated as tissue engineering scaffolds. The fibrin mesh made from fibrinogen and thrombin was also used as injectable matrices for chondrocyte transplantation since it can cross-link with native tissue in situ [19]. In general, the polymeric materials should be rationally designed depending on target applications. For example, the molecular weight should be controlled under a certain value for a controlled-delivery device, and the desired polymer structure needs to be selected for the fabrication of fibers or microcapsules because most materials can not be produced in arbitrary shapes [20].

Effects of Surface Geometry

Recent studies found that cell growth and function were closely related to the surface geometry of substrata by using micropatterned or microgrooved surfaces. Cell shape can be controlled as spreading or round by controlling the size of the adhesive island on the μm scale using the PDMS (polydimethylsiloxane) stamp to pattern the surface or microcontact printing technique. The spreading cells on the larger islands (> 30 μm) tend to grow faster while the round cells on the small island entered either apoptosis or differentiation for capillary endothelial cells or hepatocytes, respectively [21-23]. These studies determined the critical role of cell shape on the switch from growth to apoptosis or differentiated state. Microgrooved surfaces with different groove depth and width not only influenced the cell shape but also the orientation of skin fibroblasts along the groove, providing the contact guidance of cells cultured on the microtextured surface [24,25]. In addition, groove surface (rough surface) had significant effects on improving cell adhesion and tissue ingrowth [26]. Surface roughness indicates random geometrical configurations in contrast to regular geometry such as micropatterned surfaces as discussed above. Different cell types have different responses to surface roughness. Fibroblasts and epithelial cells prefer smooth surfaces while more osteoblast-like cells attach to rougher surfaces. However, the cell maturation state was more important than cell types under certain conditions. Immature or mature osteoblast-like cells can respond differently to surface roughness [7].

Biomimetic and Bioactive Surface

Recent studies also focused on creating biomimetic surface by immobilizing functional ECM protein fragment onto the surface. The biomimetic surface with peptide sequences Arg-Gly-Asp (RGD) promoted osteoblast-like cell spreading and adhesion, cytoskeleton (F-actin) and focal contact formation, and thus cell proliferation [27]. The complex micropattern technique was used to create heterogeneous surfaces that were capable of inducing selective cell attachment in coculture of hepatocytes and fibroblasts [28]. A biomimetic, micropatterned surface was created by immobilizing and spatially distributing fibronectin fragment RGD or laminin fragment IKVAK onto patterned PLA-PEG-biotin surface to control cell organization. A directional control over nerve regeneration and endothelial cell spreading were achieved on this surface [29]. The functionality of PLA-PEG-biotin surface is universal for most ligands because biotin moiety provides a universal linkage for surface engineering, while most surface-modifying techniques are only specific to a certain class of ligands [30]. Recent developments incorporated various growth factors with micropatterned surfaces. Photoreactive EGF (epidermal growth factor) conjugate was immobilized on a micropatterned surface and the pattern-immobilized EGF transduced the signal through a cognate receptor to stimulate growth of the cells with overexpression of EGF receptors. It was found that the biological signal was transduced only to the cells that interact with immobilized EGF [31].

THREE-DIMENSIONAL SCAFFOLDS

Tissue culture using 3-D scaffolds more resembles native tissue environment compared to 2-D culture. The advantages of 3-D culture over 2-D culture are summarized in Table 2. Generally, 3-D culture systems

Table 2. Comparison of 2-D and 3-D cultures

Culture method	2-D culture	3-D culture
Culture condition	Petri dishes, tissue culture flasks, multiwell plates, PLGA, PGA, PLLA films.	Microcarrier, foam or fibrous matrix fabricated from natural or synthetic polymer.
Cell morphology	Monolayer usually. But multilayer also exists depending on cell type. After confluence, cells either lift from surface or maintain postconfluent state.	In microcarrier, cells develop from monolayer to multilayer. Form aggregates. 3-D organization Quiescent cells in the inner regions of the aggregate were differentiated. May exist necrotic center.
Characteristics	1. Inadequate to model the complex cellular interactions which promote tissue-specific differentiation. 2. Rapidly lose their differentiated phenotype. 3. Expose to more hydrodynamic damage. 4. Less drug resistance.	1. Phenotypic diversity maintained in 3-D structure such as cell-cell interactions. 2. 3-D structures provide protection from the shear force and effect of air-liquid interface. 3. 3-D provide more surface area per unit volume of liquid. 4. More resistant to cytotoxic agents. 5. Transport limitations of oxygen and nutrients may lead to necrotic center.
Proliferation	After confluent, growth reached a plateau and cells begin to lift from the surface. Or, growth continued and then reached a plateau. Multilayer was formed.	Longer proliferating phase. Have the potential to yield higher final cell number.
Differentiation	Alkaline phosphatase activity no longer increased and reached plateau quickly. Per cell production of differentiated characteristic protein decreased	Maintain or promote differentiation activity. For example, alkaline phosphatase activity increases fast; 3-D induce osteocalcin synthesis, but 2-D does not in bone engineering

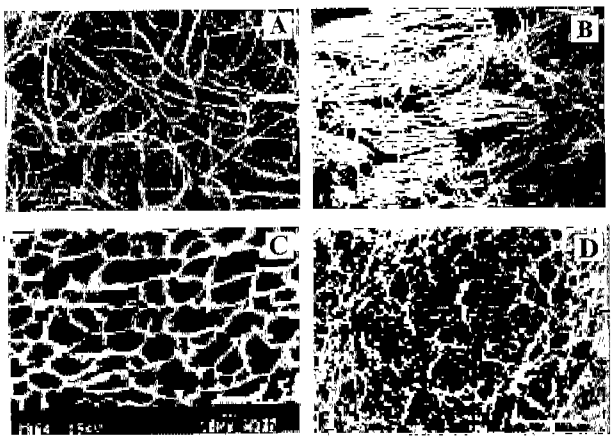

Fig. 2. Comparison of the pore structure of various tissue engineering scaffolds (SEM images). (A) nonwoven PET fibrous matrix; (B) knitted PET fibrous matrix with oriented strand; (C) polylactide (PLA) foam with oriented pores (reference [77]); (D) fibrin gel (reference [67]).

sustain high cell density, a longer proliferation period and progressively increased differentiation activity, while in 2-D culture, the proliferation is limited due to the small available surface area and the differentiation activity may be lost after a certain culture period. Morphologically, 3-D systems promote aggregate formation and 3-D cell-cell interactions, which determine the expression of cell specific function. For example, chondrocytes cultured in 2-D Petri dishes dedifferentiated, while in three dimensions, they maintained their differentiated phenotype and function [19]. It was found that the expression of various ECM proteins and their receptors in 3-D cell culture reflected the *in vivo* situation much better than that in monolayer culture [32]. In addition, 3-D cellular configuration could be more drug-resistant than a monolayer of the same cells by forming compact aggregates [33]. As the key component in 3-D culture systems, various types of 3-D scaffolds have been developed in recent years. The appropriate selection of scaffold for different applications is an important strategy in tissue engineering study. In general, the scaffolds can be classified as foam-like structures, including porous sponges and porous gels, and fibrous (woven, knitted and nonwoven) structures. Fig. 2A is an example of nonwoven fibrous matrix, which is composed of randomly distributed fibers. A knitted fibrous matrix is shown in Fig. 2B, revealing the oriented fiber bundles. A PLA foam with oriented pores and a fibrin gel with small pore sizes (~ 1 μm) are shown in Figs 2C and 2D, respectively. The differences in structures and characteristics of these scaffolds are discussed next.

Foam-like Scaffolds

Most 3-D scaffolds are fabricated as foams, including porous sponges and porous gels, which have geometric spherical pores ranging from 1 μm to 800 μm in diameter. These foams and gels are usually made from materials such as PGA, PLA, PGLA, PLGA, collagen, gelatin, agarose, alginate, fibrin, and chitosan. The pore size ranges and applications of different foams, sponges, and gels are summarized in Table 3. The porous cellulose beads have also been used as tissue engineering scaffolds in culturing hepatocytes. Hybrid foams can be fabricated from two different materials, such as collagen and PLGA, to achieve a desired mechanical property and to improve cell adhesion [34]. The three-dimensional structures of these foams and sponges are usually isotropic and easy to fabricate and quantify. Some are open pore structures and some are closed pore structures with different interconnectivity. The porosity is usually low and less than 90%. For the same materials, porous sponges with continuous solid phase can resist larger compressive loads than unbonded fiber-based scaffolds [35]. Pores with non-spherical configurations found in non-isotropic scaffolds also can be fabricated for different applications. Non-isotropic scaffolds include matrices with tubular/cylindrical pores and planar porous membranes. Foam and hydrogel scaffolds are also easy to load with drugs for controlled drug delivery.

Nonwoven Fibrous Matrices

Table 4 lists various types of fibrous matrices and their applications as tissue scaffolds. Depending on their structure, fibrous matrices can be nonwoven, woven or knitted. Woven and knitted scaffolds are not isotropic, and growth on these scaffolds more resembles those on 2-D substrates. Nonwoven fibrous matrices are usually made from either biodegradable polymers, such as PGA, PLA, and collagen, or non-biodegradable polymers, such as PET, polypropylene, polyethylene, and carbon. Nonwoven matrices are extensively used in engineering various tissues such as cartilage, bone, liver tissue, and smooth muscle. PGA-fiber-based scaffolds are also easily shaped into a variety of configurations as mentioned in the Introduction section. The structure of nonwoven fabrics highly depends on fabrication method. It is important to select nonwoven scaffolds with homogeneous fiber distribution because the non-uniform fiber distribution would result in few cells in areas that are too loose and cell aggregates clotting in areas with too densely packed fibers. The favorable distance between fibers was suggested to be 50-100 μm [36]. Needle-punched nonwoven matrices have isotropic structure, and good thermal and chemical stability in three dimensions [37]. So they are preferred as tissue engineering scaffolds. Compared to foam-like scaffolds, nonwoven matrices have a higher porosity, usually more than 90%. Currently, most commercially available biodegradable nonwoven scaffolds have similar dimension and porosity (97%), with large pores (> 200 μm). Unlike foam-like scaffolds, which are available with a wide range of pore size, nonwoven matrices are generally used in tissue culture with little control over pore size distribution Also, most biodegradable nonwoven scaffolds are incapable of resisting large compressive loads [35].

Table 3. Foam, gel and sponge scaffolds used in tissue engineering

Materials	Processing techniques	Pore size	Applications	Reference
Alginate sponge	Gelation – freezing – lyophilization	30 - 300 μm 100 - 150 μm	Fibroblast Liver tissue	[80] [18]
Chitosan	Freezing – lyophilization	1 - 250 μm	Wound dressing Dermal fibroblast	[15]
Collagen gel, foam, sponge	Freeze drying	20 and 80 μm 10, 18, 82 μm	Cartilage Liver tissue	[53] [51]
Ceramics	—	300 and 565 μm	Bone ingrowth	[61]
Cellulose bead	—	35 and 100 μm 100 and 500 μm	Liver tissue Bone marrow stroma	[71] [52]
Fibrin gel	Gelation	~ 1 μm	Cartilage Glyoblastoma Nerve outgrowth	[63] [67]
Porous hydroxyapatite	—	50 - 500 μm	Osteoconduction	[60]
Gelatin or agarose hydrogel	Freeze-drying (water as porogen)	40 - 400 μm	Neutrite extension	[42] [58]
PGLA foam	Phase separation	3 - 67 μm	Liver tissue	[50]
PLA foam PLA conduit	Hydrocarbon template Solvent casting, extrusion / particulate leaching	300 - 500 μm 250 - 400 μm 12.1 μm	Cartilage Liver tissue Nerve regeneration	[3] [72] [57]
PLGA/PEG foam	Solvent casting / particulate leaching	71 - 154 μm	Skin, intestine	[38]
PLG foam PLGA foam	Emulsion freeze-drying Solvent casting / particulate leaching Gas foaming	16 and 32 μm 150 - 710 μm 135 - 633 μm	Bone ingrowth Bone Adipose Smooth muscle	[62] [69] [59] [40]

Table 4. Fibrous scaffolds used in tissue engineering

Materials	Fiber diameter	Sp. surface area	Pore size	Applications	Reference
Carbon fiber, woven	—	—	—	Knee defect repair	[95]
Collagen fiber	—	—	25 - 125 μm	Dermal and epidermal cells	[56]
Hyaluronic acid benzyl ester, nonwoven	~ 20 μm	—	—	Cartilage	[17]
Nylon mesh, knitted and woven	— ~ 90 μm	— —	~ 90 μm ~ 210 μm	Cartilage Bone marrow stroma	[96] [86]
PET, nonwoven	~ 20 μm — —	~ 200 cm^2/cm^3 — —	30 - 40 μm — —	Trophoblast Fibroblast Abdominal wall repair	[65] [44] [97]
Polyethylene mesh	~ 400 μm	—	—	Cartilage	[98]
Polypropylene mesh	— 2.6 - 26 μm	—	—	Abdominal wall repair In vivo immune response	[97] [48]
Polyester, nonwoven	—	~ 13 cm^2/cm^3	—	Bone marrow	[90]
PLA mesh, knitted	80 - 90 μm	—	—	Bone	[82]
PGA mesh, nonwoven PLA mesh, nonwoven	~ 13 μm ~ 13 μm	~ 500 cm^2/cm^3	> 200 μm	Cartilage Smooth muscle Cardiac muscle Liver tissue Bone Intestine Bladder Artery Hybrid tissue (smooth muscle and cartilage)	[68] [11] [78] [99] [78] [35] [4] [5] [100]

Table 5. Comparison of foam-like scaffolds and fibrous scaffolds

Characteristics	Foam-like scaffolds	Fibrous scaffolds
Pore configuration	Have geometric pore	Have no geometric pore but pseudo-pore
Materials	Natural or synthetic polymer	Mainly synthetic polymer
Advantages	Easy to be fabricated and quantified; easy to control pore size (1–800 μm) and pore shape; structure and materials more diverse.	Higher surface to volume ratio; More conducive to nutrient delivery and waste removal, high pore interconnectivity.
Disadvantages	Low surface to volume ratio; gels have low mechanical strength; pore interconnectivity is limited.	Difficult and expensive to fabricate for biodegradable PLA, PGA; structure is less diverse; only limited materials can be fabricated into fibers; usually pore size is very large (>200 μm).
Applications	Have been used in various types of tissues; are more suitable for drug delivery.	Have been used in various types of tissues; are better when culturing bone marrow stromal cell, cartilage.

Processing Techniques and Structure Characterization

The traditional techniques for processing foam-like scaffolds with controlled porosity and pore size distribution are solvent casting and particulate leaching. By adjusting PLGA copolymer ratio, PLGA/PEG blend ratio, initial salt weight fraction, and salt particle size, the pliability and pore morphology can be tailored according to the requirements of a particular tissue [38]. Various new processing techniques have been developed to better control pore size and porosity of foam-like scaffolds together with the development of new biomaterials and more diversified applications. Those techniques include membrane lamination, phase separation [39], gas foaming [40], emulsion freezing/drying [41], and hydrogel freezing/drying [15,42]. The pore size and porosity were usually controlled by freezing temperature, the amount and the size of salt particles or ice crystals, and the fraction of disperse phase [39,41]. Using hydrocarbon particulate phase, the limitation on foam thickness was eliminated and the scaffolds with complex geometry can be engineered [3]. The processing technique for nonwoven matrices that is able to control pore size and porosity has not been well developed. Some modifications have been made in biodegradable nonwoven matrices to improve mechanical property. A secondary PLA or PLGA polymer can be bonded at the cross-points of the PGA fiber to improve compressional modulus and maintain the tubular shape of PGA nonwoven matrices [43]. Recently, a thermal compression technique was developed to precisely control the porosity and pore size of nonwoven PET matrices, utilizing the viscoelastic behavior of polymers. Temperature, compression pressure, and duration can be adjusted to achieve a specified porosity and pore size distribution in nonwoven PET matrices [44].

Nonwoven matrices have no geometric pores, thus the quantification of pore structure is more difficult than foam-like scaffolds. Scanning electron microscopy analysis can be used to analyze the pore structure of foam-like scaffolds or hydrogels, but is less suitable for nonwoven matrices [45]. Traditional mercury porosimetry is more suitable for hard matrices like ceramics because high pressure may be involved. Recently, a liquid extrusion method was developed to quantify the effective radius (one half of a minimum distance between surfaces within a pore formed within a fiber network) and pore size distribution of nonwoven matrices [44]. This method is especially suitable for soft textiles because a pseudo-pore theory is applied and the mild pressure range will not distort the pore structure.

The comparison of foam-like scaffolds and fibrous scaffolds is summarized in Table 5. Briefly, foam-like scaffold is easily fabricated with pore size control, and can be made from various materials. But it has low porosity and pore connectivity. So the diffusion constraints may be a limiting factor. Fibrous scaffold is less diverse in pore structure and materials. But it has high surface area to volume ratio and the transport limitation is minimal. Using foam-like scaffold or fibrous scaffold depends on the requirement of applications.

EFFECTS OF MICROSTRUCTURE OF 3-D SCAFFOLDS ON CELLULAR EVENTS

Besides cell-surface interactions in a 3-D matrix, various other properties of a 3-D scaffold can regulate tissue development, including scaffold dimension, configuration, fiber diameter, pore structure and so on (Table 6). The sandwich collagen configuration resulted in reorganization of the cytoskeleton and induced *in vivo*-like morphology and polarity of hepatocytes that was not found in monolayer culture [46]. The dimension (thickness, mm scale) of a 3-D matrix influenced chondrocyte growth rate because of the nutrient transport limitation resulting from high matrix thickness [47]. The small fiber diameter reduced cell spreading and fibrous capsule thickness because of surface curvature [48]. Of all the scaffold properties, the structure element in 3-D scaffolds was realized only recently as an essential parameter to control cell organization and functions [49]. The geometry and topology of pores in 3-D matrices can provide physical cues to modulate cellular response and guide cell morphogenesis. The

Table 6. Important properties of 3-D scaffolds and their effects on tissue development.

Properties of 3-D scaffold	Example	Tissue development	Reference
Scaffold chemistry	Smooth muscle cells in PGA, PLGA, collagen scaffolds	Phenotype shift from elastin to collagen	[11]
Scaffold pore size	Hepatocytes in PLGA and collagen foams Trophoblast in nonwoven PET	Differentiation increased with large pore size	[50,51] [65,66]
Scaffold pore configuration	Bone ingrowth Nerve regeneration in PLGA foam	Higher compressive strength in cylindrical pore than sponge pore; favorable nerve cell attachment in interconnected pore	[60] [77]
Fiber diameter	Polypropylene fiber implant in the lateral dorsum	Minimal capsule thickness and less macrophage density for small fiber diameter	[48]
Scaffold dimension	Chondrocyte in nonwoven PGA	Cell proliferation slowed with increasing thickness	[47]
Scaffold configuration	Tube shape for nerve, vascular structure; Bladder shape; Nose shape	*In vivo*-like shape promoted tissue function	[3-5]
Scaffold mechanical strength	PLA and ceramics	Bone cranial and maxillofacial surgery use ceramics; cleft palate surgery can use PLA	[82]
Scaffold biodegradability	Smooth muscle cells in PGA bond with PLA	Unbonded PGA shrink quickly and lower cell density	[81]
Hybrid scaffold	Collagen and PLGA hybrid Alginate and fibrin hybrid	Spatially uniform cell distribution; Promote chondrocyte proliferation and differentiation	[34] [101]

scaffold structure is usually expressed as porosity and pore size distribution (μm scale). The porosity, pore size, and pore configuration, as key parameters to describe pore structure of 3-D scaffolds, have profound effects on *in vitro* morphogenesis. The pore size effects of foam-like scaffold are well studied and different pore size ranges were applied to different types of tissues (Table 3). The pore size effects of nonwoven fibrous matrices, however, are less understood (Table 4). In the following reviews, the effects of microstructure of fibrous matrix on human tissue development are summarized together with the microstructure of foam, sponge, or gel.

Cell Morphology, Migration and Organization

Cell spatial organization and cell morphology determine cell proliferation and differentiation. They are directly affected by the pore size of 3-D scaffolds no matter whether a foam-like matrix or a fibrous matrix is used. Using PGLA foam as the scaffold, hepatocytes exhibited two-dimensional reorganization at subcellular foam size (3 μm), while at supercellular foam size (67 μm), 3-D aggregation was promoted [50]. A similar study was conducted using collagen foam in hepatocyte culture. At subcellular foam size (10 μm), hepatocytes assumed compact and cuboidal cell morphology. Increasing pore size to 18 μm resulted in more spreading cellular organization and discontinuous surface coverage. If the pore size was further increased to 82 μm, hepatocytes spread to a higher degree within the three-dimensional network [51]. So the effect of pore size on hepatocyte organization depended on how it was compared to cell size. Bone marrow stromal cells were cultured in cellulose beads with different pore sizes (100 and 500 μm) and in a polyester nonwoven disk [52]. Different morphologies were observed with global cells for pores of 100 μm, well-spread cells for pores of 500 μm, and needle-like cells for nonwoven disk. Different microenvironments constructed by stromal cells with different morphologies were supposed to affect hematopoiesis. For chondrocyte culture, more chondrocytes appeared as spherical chondrocytic morphology and were closely packed in collagen gel of small pore size (20 μm) compared to large pore size (80 μm) [53]. At large pore size, chondrocytes preferred to spread over the wall of the pores as elongated morphology and cell-cell interaction was limited. For fibroblast ingrowth, the optimal pore size was found to be 20 μm [41]. The pore size of 5 μm was sufficient for neovascularization because this pore size allowed the entry of host inflammatory cells [54]. However, a large pore size such as 500 μm was also successfully used for fibrovascular tissue ingrowth [55]. For adult mammalian skin, 20-125 μm were found to be the optimal pore size range. The lower limit of this pore size range was because that adequate large pore channels were required for migration of the seeded cells. The upper limit in this range was required to delay the wound contraction and to induce skin morphogenesis [56]. In nerve regeneration, fiber-based PLA conduits with small tube size for axonal growth (12 μm) successfully induced nerve tissue advancement without conduit elongation [57]. The neurite extension also depended on the pore size if 3-D hydrogel was used as scaffold [58]. The pore size range of 135-633 μm was applied to engineer adipose tissue equivalents in PLGA foam [59]. Thus, appropriate pore size ranges for differ-

ent applications were determined by the characteristics of different tissues.

The effect of pore size on bone ingrowth was studied more extensively compared to other tissues. The pore size range provided by various scaffolds in bone engineering covers 10-800 μm. However, the optimal pore size was difficult to define because different materials and pore configuration were used in different studies. In general, pore sizes of 200-400 μm were suggested to be the optimum and pore sizes of 80-100 μm were considered to be the minimum for bone ingrowth due to osteoconduction [7]. For example, examining implants from porous hydroxyapatite, cylindrical-type pores of 300 μm were found to be optimal in the whole range of 50–500 μm because more bone regeneration and higher compressive strength were observed [60]. However, it was also found that macro-pores of 565 μm were better than 300 μm using ceramics as the scaffold. More abundant bone formation was observed and homogeneous distribution in both peripheral and central macro-pores were exhibited with pores of 565 μm. Compared to the effect of macroporosity percentage, the influence of macropore size was greater in *in vivo* study of bone ingrowth [61]. As a novel approach, a PLGA scaffold with small pore size (16 and 32 μm) was designed to promote infiltration of surrounding blood, marrow, and fluid to form hematoma during bone ingrowth [62]. This small pore size scaffold successfully induced bone regeneration using the mechanism of osteopromotion and osteoinduction rather than osteoconduction. More osteoids formed in scaffolds with pore size of 32 μm than 16 μm. So the optimal pore size of the scaffolds depended on the mechanism of tissue regeneration.

Cell migration is always present during cell reorganization, and can be classified either as surface migration or 3-D migration. The occurrence of 3-D migration was largely dependent on the porosity (pore diameter) of the matrix. For example, 3-D migration was inhibited within less porous gels although cells still can migrate on the surface of the same gels [63]. Based on this fact, hyaluronan was able to stimulate migration because it increased the pore size of the fibrin network. A similar conclusion was obtained for endothelialization. Markedly retarded cell migration was found for the smallest pore (9 μm), and the endothelialization rate was higher for larger pores up to 50 μm [64]. In the hepatocyte culture discussed above, cell mobility was improved as pore size increased. At subcellular pore sizes, surface migration may exist while 3-D migration was limited. With pore size increased, 3-D migration was stimulated thus cell reorganization into 3-D aggregate was promoted. So, cell reorganization in 3-D scaffolds usually involves more 3-D migration than surface migration due to large pore size.

Since the techniques that can control pore size and porosity of a nonwoven fibrous scaffold are not well developed, the study on the pore structure of nonwoven scaffold is few. However, cell spatial organization was regulated through controlling the pore size of nonwoven

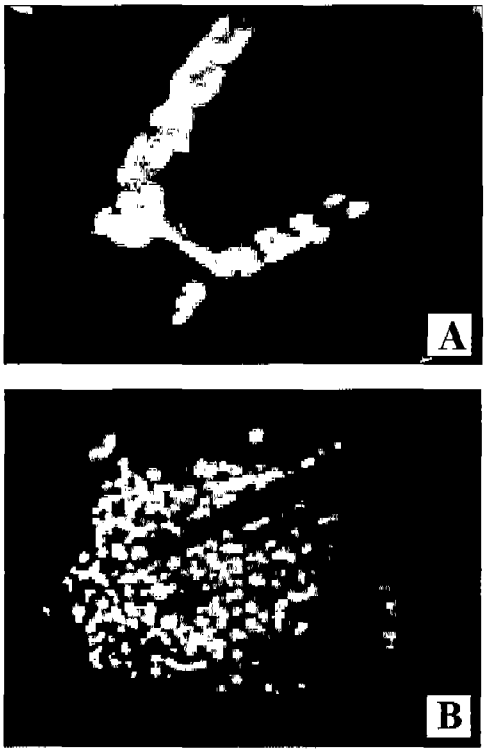

Fig. 3. Tissue engineering human trophoblast-like cells in 3-D nonwoven PET matrix (confocal propidium iodide-stained cell images, reference [65]). (A) individual cells and bridging cells in low-porosity matrix; (B) large cell aggregate in high-porosity matrix

PET matrices in a recent study of human trophoblast-like ED_{27} cells, a model cell line for studying epithelial cells [65]. In matrices with low porosity and small pore size (30 μm), cells tended to spread as individual cells on a single fiber surface or bridged across several fibers (Fig. 3A). In contrast, in matrices with high porosity and larger pore size (40 μm), cells tended to form 3-D large aggregate among the fibers (Fig. 3B). For human cord blood cells, which have a smaller cell size compared to ED_{27} cells (10 vs. 15 μm), cell aggregates can also form in low-porosity matrices together with single spreading cells (Fig. 4). So no matter whether foam-like scaffold or fibrous scaffold is used, pore structure has profound effects on cell organization and tissue regeneration.

Cell Proliferation, Differentiation, and Function

Different cell organization and cell morphology lead to different developmental patterns of proliferation and differentiation. The cell morphology with large extent of spreading promoted DNA synthesis and thus cell proliferation. In general, a low-porosity nonwoven fibrous matrix promoted individual cells spreading on single fibers, and thus cells proliferated faster than

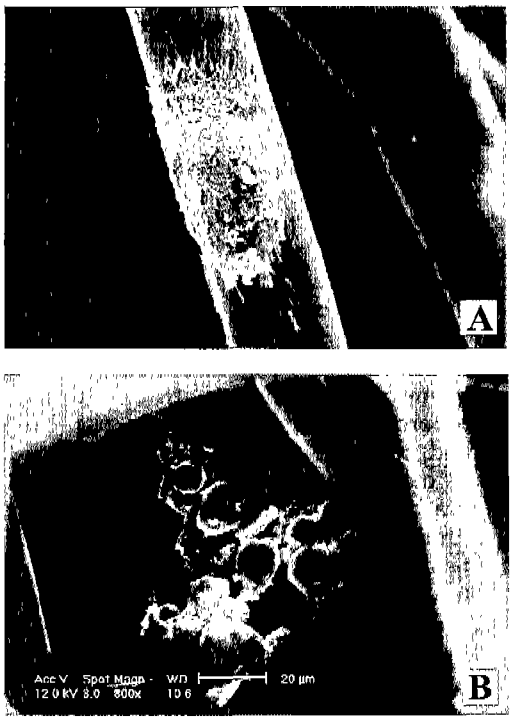

Fig. 4. Tissue engineering human cord blood cells in 3-D nonwoven PET matrix (SEM images). (A) individual cell on a single fiber. (B) cell aggregate among fibers.

those in a high-porosity matrix, as indicated by DNA content and BrdU (a DNA analog) expression [65]. Proliferation is usually not compatible with differentiation. As proliferation activity decreased, the differentiation activity was enhanced. Consistent with this conclusion, we found that large cell aggregates limited proliferation potential but promoted differentiation function as indicated by 17β-estradiol secretion from trophoblast-like cells in the high-porosity matrix. In contrast, in the low-porosity matrix, the level of 17β-estradiol secretion was significantly lower although the proliferation was faster [66]. For chondrocyte culture, in matrices that promoted spherical chondrocytic morphology and cell-cell interactions (pore size: 20 μm), the biosynthetic activity and GAG/DNA ratio were higher than spreading morphology [53]. Neurite growth decreased as fibrin density increased (*i.e.* pore size decreased), but was not influenced by the diameter or number of fibrin bundles when 3-D fibrin gel was used as the scaffold [67]. Other factors may also influence cell proliferation and differentiation under different pore sizes. It was found that chondrocytes proliferated faster and produced GAG more rapidly in nonwoven matrices with higher porosity (98% vs. 91%) and faster degradation rate (PGA vs. PLA), probably due to the nutrient availability rather than pore size itself [68]. In addition, using PGLA foam with a pore size range of 150-710 μm, no significant difference in cell proliferation and cell function (alkaline phosphatase expression) was found in osteoblast cell culture, probably because pore size in those studies covered the optimal range [69,70].

The maintenance and development of tissue function also depended on the pore structure of the culturing scaffold. The multiporous microcarriers (MCs) with small pores (35 μm) failed to immobilize enough hepatocytes and thus declined function was observed similar to floating hepatocytes in suspension culture [71]. However, MCs with large pores (100 μm) can immobilize enough hepatocytes and maintain their function [71]. Albumin secretion, an indicator of hepatocyte function, increased at all pore sizes but was the most significant at a high pore size of PGLA foam (67 μm) because 3-D aggregation was promoted [50,51]. The control over hepatocyte function was more obvious in collagen foam compared to PGLA foam. Albumin secretion rate was high (40 pg $cell^{-1}$ day^{-1}), low (3.6 pg $cell^{-1}$ day^{-1}), and medium (26 pg $cell^{-1}$ day^{-1}) at pore sizes of 10, 18, and 82 μm in collagen foams, respectively, in response to different cell morphology and organization as described above. Large pore sizes such as 250-400 μm in PLA foam or 100-150 μm in alginate sponge were also able to maintain sustained albumin secretion [18,72]. Therefore, the pore structure of fibrous matrices and foam-like matrices is a potent regulator on cell proliferation, differentiation, and tissue function.

Protein Synthesis and Gene Expression

The study of biomolecule and gene expression of cells in 3-D scaffolds with different pore sizes was few. We found that the expression of biomolecules such as cell cycle proteins was regulated by the pore size of the scaffold. Cyclin B1 and $p27^{kip}$ are biomarkers for proliferation (mitosis phase) and differentiation, respectively, and are expressed at different levels in nonwoven fibrous matrices with different pore sizes. More cyclin B1 and less $p27^{kip}$ expression were found in matrices with lower porosity and smaller pore size [66]. E-cadherin is a cell-cell adhesion protein that plays a dominant role in cell aggregate formation. Its expression was restricted to cell-cell contacts and closely relates to cell morphology, and thus to pore size and pore configuration of the scaffolds [73]. It was expressed at high level in collagen foam with a pore size of 10 μm, but depolarized in cells cultured on foam with pore sizes of 18 and 82 μm. The cytoskeleton protein vinculin was located in the shape of peripheral ring for pore size of 10 μm while it redistributed throughout the ventral plasma membranes for pore sizes of 18 and 82 μm [51].

The enhancement of gene expression may arise from pore-induced compaction in cell shape and the establishment of a high degree of cell-cell contact. The most suggestive example is liver-specific gene expression. It was postulated that a critical level of intercellular contacts might be required to elevate gene expression, and cell-cell versus cell-matrix interactions could regulate gene expression [51]. The albumin mRNA level was found to parallel with albumin synthesis and the cells with the morphology that promoted albumin secretion

expressed high albumin mRNA. In fact, the scaffold configuration was postulated to alter liver-specific gene expression via the activation of the transcription factors such as eE-TF and eH-TF [46]. Gene expression was also regulated by scaffold chemistry besides scaffold structure. The elastin gene expression was switched to collagen gene expression in smooth muscle cells when the scaffold was switched from PGA nonwoven matrix to type I collagen sponge. Similar results were observed for cells cultured on 2-D PGA or collagen films [11].

A recent study incorporated plasmid DNA into PLGA copolymer foam and then delivered tissue-inductive protein in a controllable mode after transplantation. The copolymer ratio of the matrices had profound effects on DNA release rate [74]. Similarly, growth factors such as VEGF were incorporated into PGA-PLGA copolymers by applying microsphere drug delivery technology in order to induce angiogenesis of large smooth muscle tissue. This approach was capable of overcoming the mass transport limitation [75]. The biological activity of the growth factor may depend not only on the identity of growth factor but also on how to present it to cells in space and time. Enzymes such as Factor XIIIa can be covalently conjugated into fibrin network during coagulation [76]. Similar study can be performed using nonwoven fibrous matrices. The effects of pore size and porosity of the matrices on DNA and growth factor release have not been investigated in the above studies, but they may play an important role in the release process, and need to be explored in the future.

Effects of Pore Configurations

Most studies on optimum pore size for various engineered tissues used 3-D scaffolds with random pore geometry, so the effect of pore geometry was taken into account in the effect of pore size. The specific study on pore configuration indicated that the interconnection and pore arrangement affected the histological response of bone ingrowth [60]. Cylindrical-type pores induced new bone vessels arranged in a longitudinal pattern along the pore, while in the sponge- and cross- type pores, an irregular woven bone pattern was formed. In terms of biomechanics, cylindrical type scaffolds had higher compressive strength than sponge type and were more applicable in clinics. For peripheral nerve regeneration, isotropic interconnected pore structure was favorable for cell attachment. In contrast, anisotropic foams composed of tubular pores showed only a few cells on the surface, but they were favorable to guide the axonal growth into the macropores [77]. Other tissues such as intestine and blood vessels prefer tubular scaffolds. Foam-like and nonwoven fibrous scaffolds are different in pore configuration. Higher cell growth rate and GAG deposition were observed for chondrocytes grown in PGA nonwoven mesh compared to PLA sponge, thus PGA mesh was selected as the better scaffold in cartilage engineering [78]. Clearly, pore configuration is also an important factor in regulating tissue regeneration, and should be considered separately from pore size.

Effect of Cells on Scaffold Structure

The interaction of seeded cells with 3-D scaffolds is mutual for biodegradable polymers, and cultured cells can affect the structure of the scaffold as well. It was reported that unseeded PLGA foams maintained their foam size but cell seeded foams shrank and the foam volume decreased due to the contractile forces from adherent osteoblastic cells [79]. Similarly, fibroblasts cultured on collagen foam contracted the foam down to 40% of initial volume after 5 weeks [80]. To maintain the configuration, dimension and the predefined structure of 3-D scaffolds during tissue development, PGA nonwoven mesh was bonded with PLA to resist the contractile forces from smooth muscle cells [81]. Using this method, the bonded PGA matrices degraded more slowly and maintained their volume, at the same time exhibiting a high cell density and ECM deposition. In contrast, unbonded matrices contracted to 5% of original volume and resulted in less cellularity. So the effect of cells on scaffold structure should be considered when choosing the appropriate scaffold in tissue culture.

TISSUE ENGINEERING USING FIBROUS MATRICES: CASE STUDIES

Two samples are given below to illustrate how to use the appropriate scaffold for the target applications, so that tissue structure and function are reconstructed in the way similar to *in vivo*. The challenges remained for tissue engineers are also briefly discussed.

Bone Tissue Engineering

Following skin and cartilage, bone will become the next commercial product of human tissue substitutes. The optimized scaffold for tissue engineering bone is not determined and it highly depends on the application and implantation site [82]. For applications requiring high strength (*e.g.* cranial and maxillofacial surgery), scaffolds like bioceramics and hydroxyapatite are usually applied. For applications that high strength is not the desired feature (*e.g.* cleft palate surgery), flexible devices are applied. In general, the scaffold should have the required mechanical property to sustain the *in vivo* stresses and loading, or at least allow the engineered tissue to develop sufficient mechanical integrity until it can support itself under compressive loading [83]. Because nonwoven fibrous matrices usually do not possess the required mechanical property, they should allow the development of self-supported bone tissue with sufficient mechanical strength. As an alternative strategy, knitted PLA mesh was attached to a PLA plate with fibrous surface to enhance bone growth and with the plate to retain the strength [82]. In another study, PGA nonwoven mesh was bonded with PLA to reinforce mechanical integrity [84]. Scaffolds can be loaded with bone morphogenetic proteins (BMP) so that the release of BMP can promote osteogenesis in a controlled man-

ner [85]. In addition, bone requires a relatively high rate of mass transfer. Usually, bone-like tissues with mineralized surfaces were produced on PGA meshes with tissue thickness less than 0.5 mm [78]. So it is necessary to induce angiogenesis and vascular invasion into cartilage prior to osteogenesis. Using new strategies of scaffold design, fibrous scaffolds can be successfully applied in bone tissue engineering.

Tissue Engineering Hematopoietic and Embryonic Stem Cells

Stem cells provide a novel cell source for tissue engineering. Compared to other types of stem cells including mesenchymal or neuron stem cells, hematopoietic stem cells are the most extensively studied cells, but mainly in suspension culture or 2-D Dexter culture. A few 3-D culture systems for hematopoietic stem cells were developed in recent years. A collagen-pre-coated nylon mesh was used as the template to grow rat bone marrow cells [86]. The ability of multi-lineage differentiation was maintained but the progenitor number declined with time. Porous collagen microspheres were used as the supporting matrix for murine bone marrow cell growth [87]. This system also supported multilineal development while very few progenitors were produced, probably because of unknown substances from collagen microspheres. A tantalum-coated porous biomaterial (CellFoam) was found to support marrow $CD34^+$ cell expansion up to 1.5 fold in the absence of cytokines, due to the feature of 3-D microenvironment [88]. However, the microenvironment in CellFoam was unclear because the inorganic materials can not be cut or visualized using techniques for 3-D study. Recently, a nonwoven PET matrix was employed as a biocompatible scaffold for the growth of human cord blood cells (Figure 4). The 3-D structure of bone marrow microenvironment was simulated in an attempt to promoting self-renewal and differentiation of hematopoietic progenitors in a controlled manner [89]. Nonwoven fibrous matrices with controlled pore size (10-60 μm) used in the above study provided isotropic structure and 3-D local environment compared to the porous structure of collagen spheres or CellFoam with large pores of 200-300 μm. Compared to 2-D $CD34^+$ cell culture, 3-D culture supported 2-3 fold higher total cell number and progenitor cell number for 7-9 weeks. Culture in 3-D nonwoven matrices enhanced cell-cell and cell-matrix interactions and allowed spatial distribution of stromal and hematopoietic cells, indicating that the spatial microenvironment in the nonwoven matrix played an important role in promoting progenitor production. In contrast, cord blood cells grown in fibrin gel were induced to differentiation, and progenitor number declined after 5 weeks (unpublished results). The advantage of nonwoven polyester matrix was also reported for bone marrow cell culture in constructing 3-D hematopoietic microenvironment [90]. Compared to porous cellulose beads (pore size of 100 and 500 μm), stromal cells on nonwoven disk supported the self-renewal of progenitors while those on cellulose beads did not, due to the physical structure of the nonwoven matrix. So the fibrous matrix demonstrated its superiority in hematopoietic culture over other 3-D matrices.

Embryonic stem cells have the potential to differentiate into any type of tissue cells and provide new models for understanding the basic processes that control human tissue development [91]. The challenge for embryonic stem cell research is to direct the stem cells into a specific lineage rather than a mixture of various tissue cells. For example, a stromal cell and embryonic stem cell co-culture system was developed to induce the differentiation of embryonic stem cells into hematopoietic cells [92]. Currently, the chemical parameters that regulate the developmental pathway of embryonic stem cells are studied, especially focusing on various cytokines that can induce the differentiation of different tissue types [93,94]. The use of 3-D scaffold, including fibrous matrices, and the effects of their physical parameters on tissue development have not been investigated yet, and these need to be investigated in future study.

Challenges and Future Directions

There are still many remaining challenges and problems to address in furthering tissue engineering for clinical applications. The most important issue is how to achieve a uniform cell distribution in scaffolds with defined microstructure. Most current cell-polymer constructs are not uniformly distributed, which deviates from the *in vivo* tissue and may result in an inferior tissue construct. The next question is how to engineer mixed types of tissues in scaffolds with large dimensions that are capable of angiogenesis. The co-culture of different tissue cells is required toward organ engineering. With the increase of cell density and construct dimensions, the nutrient delivery by the self–supported vascular structure is essential. Also, more studies are necessary to better understand how the pore structure of 3-D scaffolds regulates gene expression. Lastly, using embryonic stem cells to derive various tissue types provides a promising new approach in tissue engineering. However, much less is known in using embryonic stem cells as cell source, especially in understanding how the physio-chemical environment regulates and directs the differentiation of stem cells.

CONCLUSION

The 3-D tissue scaffold plays a critical role in regulating human tissue development. Besides the usual properties important to 2-D substrata, including surface chemistry, geometry, and bioactivity, the pore structure of a 3-D scaffold is also an important parameter in controlling cell organization, proliferation, differentiation, and thus tissue development. The use of foam-like or fibrous scaffolds depends on the requirement of different applications. The study on the significance of 3-D

scaffolds and their pore structure, however, is far from completion. Most current studies are limited at the observation level. The underlying mechanism of pore size regulation in tissue development has not been well understood. The effect of pore structure may be due to the altered gene expression, the cell size, or the mechanism of tissue regeneration. However, these postulations need to be further proved, and studies at the protein and molecular levels would be helpful. Using new approaches, such as embryonic stem cells as cell source and novel design of 3-D scaffolds, tissue engineering brings a promising future to patients requiring tissue repair and organ replacement.

ABBREVIATIONS

ALP : alkaline phosphatase
BMP : bone morphogenetic protein
ECM : extracellular matrix
EGF : epidermal growth factor
GAG : glycosaminoglycans
PEG : poly(ethylene glycol)
PET : polyethylene terephthalate
PGA : poly(glycolic acid)
PLA : poly(lactic acid)
PGLA : poly(glycolic-co-lactic acid)
PLG : poly(lactic-co-glycolide)
PLGA : poly(lactic-co-glycolic acid)
PTFE : polytetrafluoroethylene
VEGF : vascular endothelial growth factor

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