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Micro- and Nanoscale Control of Cellular Environment for Tissue Engineering

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18.1
Overview

Tissue engineering is a potentially powerful approach for restoring organ functionality and overcoming the shortage of transplantable organs. In tissue engineering, the principles of engineering and life sciences are used to develop biological substitutes, typically composed of biological and synthetic components that restore, maintain, or improve tissue function [1]. Although relatively simple tissues such as cartilage and skin have already been successfully engineered, many basic challenges persist in the engineering of more complex tissues. These challenges – which include the generation of vascularized tissues and complex geometries – can be traced to our limited abilities to control the cellular environment at micro- and nanoscale resolution. Cells in the body reside in an environment that is regulated by cell–cell, cell–extracellular matrix (ECM) and cell–soluble factor interactions presented in a spatially and temporally dependent manner (Figure 18.1). In order for tissue engineering to succeed, it is critical to reproduce these *in-vivo* factors outside the body. In this chapter we analyze the use of micro- and nanoscale engineering techniques for controlling and studying cell–cell, cell–substrate and cell–soluble factor interactions, as well as for fabricating organs with controlled architecture and resolution.

18.1.1
Cell–Substrate Interactions

Decisions such as cell growth, migration, and differentiation can all be affected by a cell's interaction with the surrounding surfaces. Numerous micro- and nanoengineering approaches have been used to control cell–substrate interactions *in vitro* through presenting specific molecules to cells. These molecules, which

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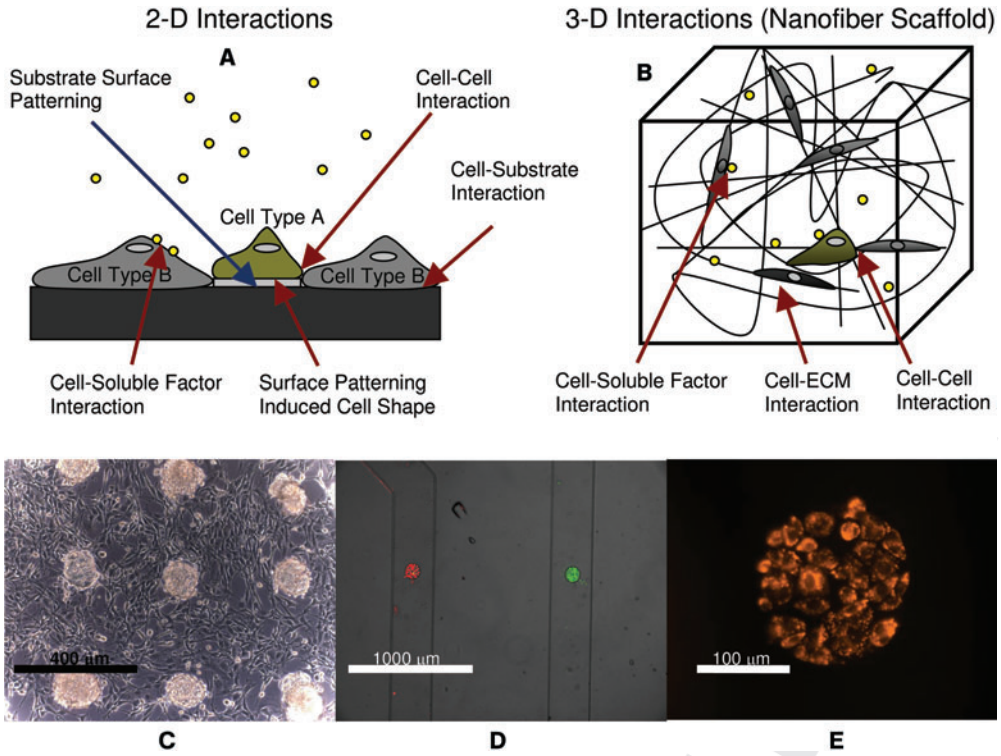

Fig. 18.1 Cell–microenvironment interactions for tissue engineering. The upper row depicts a schematic of interactions in (left) two dimensions (2-D) and (right) three dimensions (3-D). The lower row provides examples of: (c) patterned co-cultures; (d) cells within microfluidic channels; and (e) cells that are micropatterned on a substrate.

range from adsorbed protein, to engineered peptides, to non-adhesive polymers, can be used to engineer many cellular functions *in vitro*. For example, self-assembled monolayers of alkanethiols have been shown to affect the action potential generation capacity of neurons [2], as well as the differentiation and proliferation of myoblasts [3]. Self-assembled multi-layer structures with cholesteryl moieties have also been shown to improve fibroblast adhesion and spreading [4]. Alternatively, surfaces can be engineered to prevent protein adsorption and cell adhesion on a variety of surfaces [5].

Many biological processes such as cell migration, axon extension and angiogenesis are regulated by spatially dependent signals, including surface gradients of molecules. Traditional macroscale techniques have been limited in their ability to form spatially regulated patterns of molecules. Micro- and nanoengineering

approaches can be used to generate *in-vitro* gradients of molecules on substrates to mimic natural environments. For example, surface gradients of laminin have been generated using a microfluidics system and have been used to study the extension of axons under controlled conditions [6]. Alternatively, microscale gradients of surface molecules [7] were engineered into planar surfaces by merging the gradient generation capacity of microfluidics and photopolymerization chemistry. Using this technique, gradients of the adhesive peptide RGDs (Arg-Gly-Asp-Ser) were formed and could be used to study cell adhesion [8]. The gradients of hydrogels with varying polymer concentrations can also be used to study the response of cells to surface stiffness.

Micro- and nano-textured substrates have also been shown significantly to influence cell adhesion, gene expression [9–11], and migration [12]. Nanoengineered topographies can be incorporated into tissue engineering scaffolds to provide functional cues to cells. In fact, surface texture has already proven to be an important parameter in current orthopedic replacement/augmentation applications [13]. It is generally believed that implantable orthopedic materials must be hospitable for osteoblasts which deposit new bone matrix directly adjacent to the material. As such, many investigations in the filed of orthopedics have focused on the fabrication of biomaterials which maximize cellular adhesion, and indeed it has been found that nanotextured materials which mimic the nanoscale features of bone surfaces are especially suitable for these applications [13]. It is likely that the superior adhesive properties of textured materials are due to the increased particle boundaries and increased surface area available for osteoblast adhesion. Such nanoscale features, which are typically less than 100 nm, may be created through a number of techniques, such as chemical etching and anodization. These techniques have proven to be especially efficient for generating surface roughness on metals such as titanium, and it has been shown that nanotextured surfaces prepared by chemical etching [14] induce significantly higher levels of metabolic activity.

Another approach for generating nanotopography is to embed nanoscale objects within biomaterials. For example, carbon nanofiber-embedded composites have been generated with increased osteoblast adhesion and decreased adhesion for other cell types [15]. The decreased adhesion for other cell types is especially advantageous in that it reduces the formation of disruptive fibrous tissue around the implant. Another alternative technique that is widely applicable to a range of materials (e.g., metals, ceramics, carbon fibers, composites) is the use of nanoparticles (NPs) as composite materials [16–19]. A detailed review of such technologies is beyond the scope of this chapter, but for the interested reader more comprehensive reviews of nanotextured materials [13] and cell–surface interactions [20] can be found elsewhere.

One potential advantage of micro- and nanoscale technologies is that they miniaturize experiments and can be used to perform high-throughput analysis. For example, robotic spotters can be used to perform high-throughput analysis of cell–substrate interactions. In these studies, stem cells were interfaced with thou-

sands of different polymeric materials that were patterned using microarray technology. The effects were observed for human embryonic stem (ES) cells [21] and human mesenchymal stem cells (hMSCs) [22], and resulted in the identification of some surprising cell–material interactions. Although the specific biological mechanisms underlying these interactions are not known, this method can be used to identify novel and potentially useful biomaterials, and for monitoring unexpected cell responses. A variation of this approach has also been used to analyze cell differentiation in response to combinations of ECM molecules [23].

18.1.2 Cell Shape

The effect of cell shape on various cell fate decisions has been studied by patterning cells onto micropatterned substrates. Differences in the size and shape of the adhesive region cause cytoskeletal rearrangements, which have been shown to effect proliferation and apoptosis [5]. For example, smooth muscle cells and endothelial cells [24–26], when micropatterned on poly(lactic-*co*-glycolic acid) (PLGA) surfaces, and have been shown to provide a better maintenance of cell function and morphology. In addition, cell shape has been shown to direct stem cell differentiation. Human MSCs that were patterned on small and large fibronectin patterns differentiated differently based on the size of the patterns. Typically, small islands induced the cells to form spheres, while large islands induced them to flatten and to adhere to the surface. Staining for differentiation markers indicated that the spherical cells differentiated into adipocytes, while flattened cells became osteoblasts [27].

Within the body, cells are exposed to dynamic environments which may alter their shapes in response to changing mechanical forces. Typical patterning techniques generate fixed patterns which cannot be changed after the deposition of adhesive molecules. However, in order to study the dynamics of cell shape change there is a need for substrates that can be dynamically altered to regulate the adhesive cell environments. Towards this end, photoinitiated gels [28, 29] and thermally responsive polymer surfaces [30] have been developed. In addition, other techniques such as the electrochemical modulation of self-assembled monolayers [31, 32] have enabled the reversible switching of surface properties. An alternative dynamic patterning approach uses surface cracks to reversibly modulate the adhesive properties of the substrate at the scale of adhesion complexes [33]. By using this approach, adhesive signals can be patterned within nanocracks of a poly(dimethylsiloxane) (PDMS) substrate, and the availability of such signals can be reversibly modulated multiple times by varying the strain applied to the substrate. However, this technique is only capable of generating parallel nanoscale cracks, and is ineffective for the generation of more complicated nonlinear patterns. Clearly, many further studies must be carried out to develop approaches capable of generating dynamic patterns at the nanoscale, with more flexibility and precision.

18.1.3
Cell–Cell Interactions

One of the biggest challenges in tissue engineering is to reproduce *in vitro* the specific arrangement of cells found *in vivo*. The co-culture of different cell types is one approach to artificially recreate these arrangements. For example, hepatocytes in co-culture with endothelial cells better maintain their differentiated phenotype [34]. Similarly, homotypic hepatocyte cell–cell interaction, such as hepatocyte spheroids, can be used to better maintain a differentiated phenotype [35]. However, the degree of cell–cell interactions in these co-cultures cannot easily be controlled without the use of microscale technologies. In an effort to control the degree of heterotypic and homotypic cell–cell interactions, micropatterned co-cultures have been used; these are patterned through the use of micropatterned adhesive substrates which selectively position various cell types relative to each other. Patterned co-cultures were initially used to study the cell–cell interactions between hepatocytes and non-parenchymal fibroblasts [36], and have led to important findings about the nature of hepatocyte and fibroblast interactions [37–39].

Other methods for generating patterned co-cultures utilize such techniques as thermally reversible polymers [40, 41], layer-by-layer deposition of ionic polymers [42], microfluidic deposition [43], and micromolding of hydrogels [44]. Although cells have recently been patterned [45] onto three-dimensional (3-D) scaffolds using replica printing techniques, further investigations will need to be carried out on the incorporation of such fundamentally two-dimensional (2-D) techniques into 3-D tissue engineering constructs.

Beyond patterning surfaces to generate co-cultures, another approach might be physically to confine cells and cell aggregates within defined spaces. It is known that characteristics such as cellular phenotypic expression and stem cell fate decisions may be affected both by physical interaction with surfaces and by the diffusion limitations introduced by confining cells and cell aggregates within spaces. The recent use of microwells [46] for generating arrays of many different cell types is a potentially useful tool for facilitating and studying the effect of culturing and co-culturing cell types within confined spaces. Beyond its applications to the generation of high-throughput multicellular arrays, this approach may also be amenable for generating co-cultures of many cell types.

18.1.4
Cell-Soluble Factor Interactions

Soluble factors such as signaling proteins and nutrients are also important components of a cell's microenvironment. Both, micro- and nanofluidic technology can be used to control the spatial and temporal presentation of soluble factors to cells *in vitro*. For example, laminarly flowing fluids within microchannels can be used to pattern cells and their substrates [47, 48]. By using laminar flows, a cell

may be simultaneously exposed to multiple spatially segregated soluble factors [49]. Such a technique may be useful for a wide range of studies in which the delivery of spatially segregated factors or conditions are relevant and allows for characterizations of intracellular molecular kinetics. Microfluidic patterning can also be used to study the effects of soluble factors on the population of cells. For example, temperature gradients have been used to control the development of different sides of an embryo [50]. Microfluidic gradient generators have also been used to study the effects of molecular gradients on cells [51, 52]. Using this technique, neutrophils were exposed to gradients of interleukin (IL)-8 to generate a novel understanding of the migration behavior of cells in response to nonlinear gradients [53]. Microfluidic gradients have also been shown to effect neural stem cell differentiation [54] and axon extension [55, 56].

18.1.5 3-D Scaffolds

Traditionally, ''engineered tissues'' have been fabricated by seeding cells within porous 3-D scaffolds, with such scaffolds serving as an environment within which nutrient and oxygen transport, as well as mechanical support [57], are provided for cell growth and proliferation, leading to the formation of 3-D tissues. Under ideal conditions, the scaffold would gradually degrade and become replaced by the ECM molecules deposited by cells, eventually leading to 3-D structures which resemble native tissue architecture. Porous scaffolds are currently generated through a variety of processes, including solvent casting and particulate leaching [58]. Although these technologies can be potentially used to generate nanoscale structures, such processes cannot be used to engineer properties such as pore size, geometry, interconnectivity, and spatial distribution of the scaffolds. Furthermore, the diffusion limitations imposed by the scaffolds generated using current techniques has prevented the engineering of larger pieces of (more than a few hundred microns) viable tissue [59, 60].

Emerging techniques which have been used to generate scaffolds with microscale resolution include 3-D printing, microsyringe deposition, and tissue spin casting. 3-D printing (3DP) is typically used to generate ceramic [61] scaffolds in orthopedic tissue engineering applications. Similarly, in organ printing the cells and matrix material may be printed and built up [62]. Although 3DP confers a great deal of control over the macrostructure of the scaffold, like solvent casting and particulate leaching it suffers from process-derived limitations on porous properties. Alternatively, microsyringe deposition which has been used to generate layered PLGA scaffolds [63] confers a much higher degree of control, with fairly precise resolutions over the macrostructure of the scaffold, albeit at the expense of rapidity. One can imagine a combination of 3DP with microsyringe deposition which might enable the generation of much higher-resolution scaffolds. Also, the layering or combination of multiple ''pieces'' of engineered tissue is generally applicable to other materials such as polyurethane [64], and recently has been used to build larger structures using microfluidics and spin-coating ap-

proaches combined with particulate leaching [63]. Variations on these approaches are extensive, however, and the interested reader will find a more comprehensive review of such technologies elsewhere [65–67].

The aforementioned techniques have, despite their applicability to nanoscale features, only been applied to the generation of microscale features. One current approach for creating nanoscale scaffolds builds these structures from polymer nanofibers which are typically around a few hundred nanometers in diameter. Indeed, many natural biomaterials such as collagen and chitin are composed of fibrous structures. Techniques for fabricating nanofibers include electrospinning, melt-blowing, phase separation, self-assembly, and template synthesis.

Electrospinning is a popular approach as it is relatively inexpensive and capable of producing nanofibers from a variety of polymers and other materials. A number of polymers – including but not limited to PLGA, collagen, polycaprolactone (PCL), poly(L-lactic acid) (PLLA) and so forth – have been used to generate scaffolds for orthopedic [68], cartilage [69], and cardiac tissue engineering [70] applications. The resultant structures may be extremely porous and have very high surface area to volume ratios. Specific nanoscale features play a prominent role both in promoting cell proliferation and in guiding cell growth and general tissue architecture. Towards this end, recent investigations have shown that nanofibers aligned in desired directions and patterns [71] can induce growth and proliferation of cardiac cells into biologically relevant contractile spindle structures. In addition, as nanofibers can be generated from well-characterized polymers such as PLGA, it is possible to take advantage of properties such as biodegradability and surface functionalization. In fact, the size of biodegradable fibers can be used to modulate the degradation rate of the material.

Self-assembled amphiphilic peptides (Figure 18.2) were recently formed into hydrogels for tissue engineering [72] which resembled bone matrix alignments [73]. Peptide groups may be customized to direct cell behavior and polymerized directly into the hydrogel. For example, it was shown that directed differentiation of neural stem cells could be modulated using such a hydrogel functionalized

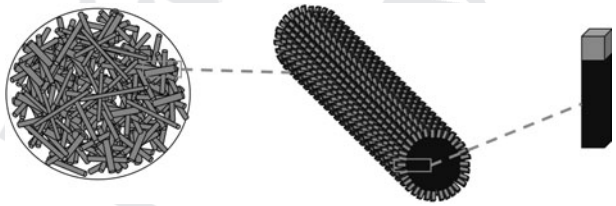

Fig. 18.2 Structure of self-assembled amphiphilic peptide hydrogels. (Left) A macroscopic hydrogel is composed of (center) fibers (which form in solution) that are in turn composed of (right) amphiphilic peptides. The hydrophobic hydrocarbon tails (black) of the peptides are buried within the fiber interior, while the hydrophilic peptide headgroups (gray) are exposed [73].

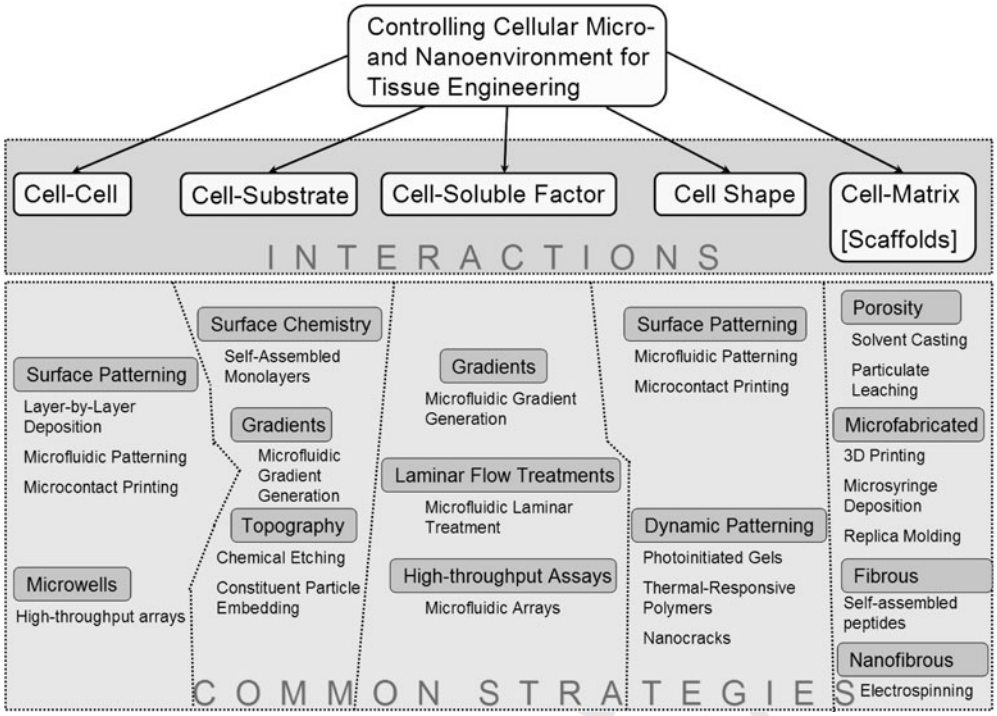

Fig. 18.3 An overview of cell–microenvironment interactions and relevant techniques.

with isoleucine-lysine-valine-alanine-valine (IKVAV, a laminin-derived sequence) without the use of additional biochemical factors [74].

18.2 Methods

The general methods and themes related to the techniques mentioned in Section 18.1 are described in the following sections (see Figure 18.3).

18.2.1 Soft Lithography

Traditional lithography, as developed by the semiconductor industry, has inspired the recent emergence of soft lithography and other fabrication techniques in the generation of micro- and nanoscale features for tissue engineering applications [75]. Soft lithography can be used to fabricate small-scale features without the use of expensive clean rooms by utilizing elastomeric molds made from patterned silicon wafers (Figure 18.4). A number of techniques for controlling the

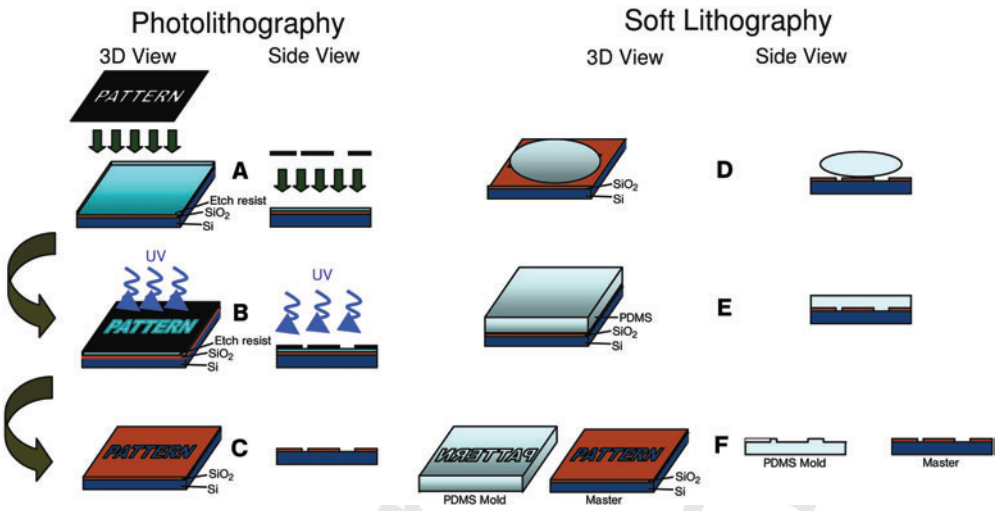

Fig. 18.4 A schematic overview of photolithography and soft lithography. In photolithography (a), a mask is placed on a silicon wafer that is coated with a thin layer of photoresist (b). The mask and wafer are exposed to ultraviolet (UV) light, which generates a patterned silicon wafer (c). In soft lithography (d), a liquid prepolymer (typically PDMS) is molded on a patterned silicon wafer/master (e). The polymer is then cured and the PDMS mold is subsequently peeled from the stamp (f).

micro- and nanoscale cellular environment rely on this approach. In particular, many micropatterning techniques for facilitating cell–cell interaction and controlling cell shape utilize microfabrication techniques. For example, techniques such as capillary force lithography, micromolding, microcontact printing, and microfluidic patterning for generating specific patterns of molecules on planar surfaces often rely on microfabrication approaches [76]. In capillary force lithography and micromolding, molds placed on a layer of prepolymer solution displace the polymer solution into the void regions of the mold before polymerization is induced [77]. In microcontact printing, molds may be "inked" with molecules and placed on top of surfaces in order to print them in the shape of the mold pattern [78]. In microfluidic patterning, molecules may be flowed through microfluidics channels produced by layering a mold on top of a flat surface [75]. Recently, the gradient generation capacity of microfluidics techniques has been coupled with photopolymerization chemistry to generate hydrogels with spatial control of the gel properties [7, 79]. In these studies, gradients of photocrosslinkable monomers were generated within microfluidics channels and then photopolymerized via exposure to ultraviolet light. In addition to creating crosslinking density gradients across a material, gradients of conjugated signaling or adhesive molecules were also generated for directing and inducing cell migration, adhesion, and differentiation.

Nanocrack structures that enable dynamic cell patterning are also made from PDMS substrates generated by soft lithography [33]. Such substrates are exposed

to oxygen plasma to ''harden'' or to create a silica-like film on the surface rendered resistant to protein adsorption via the deposition of tridecafluoro-(1,1,2,2-tetrahydrooctyl)-1-trichlorosilane. The substrates are then subjected to uniaxial mechanical strain (a combination of strains can be applied at once and in difference directions) and, due to differences in surface and bulk properties, parallel arrays of nanocracks are generated. The bulk material exposed by cracking is not resistant to protein adsorption, and so the exposure of this bulk material allows for the adsorption of ECM proteins that in turn facilitates cell attachment along these parallel lines. The crack widths can be modulated by varying the strain applied, and the crack can be closed by reducing the strain, thereby reversing the availability of adhesive portions of substrate. This technique can be applied repeatedly in different directions to dynamically direct the growth and retraction of cells along linear nanocracks.

Microfabrication approaches may further be used to generate features within 3-D scaffolds, and indeed finer structures such as microvasculature have been generated in tissue scaffolds using microfabrication approaches [80, 81]. Initially, standard photolithography techniques were used to create vascular network patterns directly upon silicon and Pyrex surfaces. Cells were seeded onto the surface and allowed to grow to confluence, and then peeled off as a monolayer and ''rolled'' together into a rough approximation of tissue. Later studies relied on soft lithographic replica molding of the patterned networks generated using these standard micromachining technologies with biocompatible materials such as PDMS, PLGA [82] and poly(glycerol sebacate) (PGS) [63]. Generally, replica molding is performed by the pouring of a mixture of prepolymer solution and crosslinking agent onto a (typically silicon) patterned surface, followed by subsequent photo- or thermally catalyzed polymerization of the mixture. Replica molding, coupled with particulate leaching, can further generate biodegradable porous microfluidics structures. For example, artificial capillary networks were generated through layer-by-layer stacking. Finally, layer-by-layer techniques may also be used with microfluidic patterning to generate cell-seeded scaffolds [83–85]. Here, cell-prepolymer solutions are repeatedly flowed, deposited, and polymerized within regions of microfluidics channels in a sequential manner. The sequential deposition of different cell-prepolymer solutions allowed for the controlled generation of layers of different cells within scaffolds.

18.2.2 Self-Assembled Monolayers

Self-assembled monolayers of alkanethiols used for cell culture may be formed on gold surfaces, either through direct or vapor deposition of alkanethiols to the surface. In this technique, a silicon or glass substrate is typically first coated through vacuum evaporation with a thin layer (e.g., 1 nm) of titanium to facilitate gold adhesion, followed by coating with gold. Upon vapor deposition of the alkanethiol,

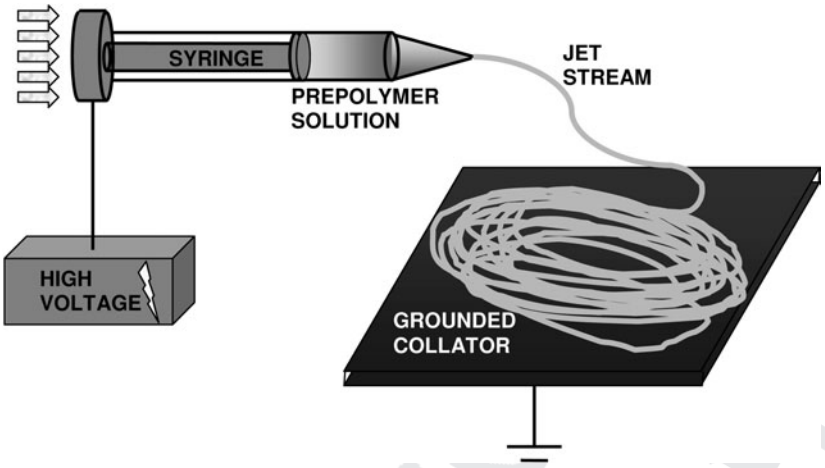

Fig. 18.5 A schematic diagram of polymer nanofiber electrospinning. A high voltage is applied to a syringe in order to charge the prepolymer solution contained within. The prepolymer solution is then ejected from the syringe in a controlled manner, and a jet stream of the charged prepolymer is drawn towards a grounded (earthed) collator. As the jet stream moves through the air towards the collator, the polymer dries to form nanofibers. The charge of the polymer slowly dissipates after collecting on the collator.

the molecules' sulfur groups will orient themselves to contact with the gold surface, while the alkyl chains are packed away from the surface.

18.2.3 Electrospinning

Electrospinning – a technique derived from electrostatic spraying – is the most common method of producing nanofiber scaffolds as it is capable of producing fibers of various sizes (from tens of nanometers up to microns) from various materials (Figure 18.5). In brief, a charged material solution (e.g., a polymer such as PLA) dissolved in a solvent is discharged from a reservoir through a tip that ranges in size from few hundred microns to a few millimeters. A grounded (earthed) collecting surface (collator) below the tip that is 10–30 cm away will draw the "whipping jet" of ejected solution towards it. As the jet travels through the air towards its target, the solvent dissolves into a nanofiber, which collects upon the grounded surface. With time, the nanofiber eventually loses its charge and the result is a mat of fiber which can be used as the basis for scaffold material. In order to achieve alignment in particular directions or dimensions, the collecting surface can translated relative to the dispensing tip. Fiber dimensions and characteristics may be controlled through modulation of material concentration in solvent. Layers of woven or non-woven mats of nanofibers may be stacked upon one another to build larger scaffold structures.

18.2.4
Nanotopography Generation

In order to generate nanoscale surface roughness, chemical etching techniques are often used. For example, a machined surface may be treated with a solution of H_2SO_4 or other corrosive solution to generate nanotextures. Alternatively, nanotextured surfaces may be generated through powder metallurgy (or the embedding of constituent NPs), which can be applied to materials such as pure titanium, Ti6A14V and Co28Cr6Mo. In this approach, powders are typically loaded into a steel-tool die and compacted at high uniaxial pressures (on the order of many GPa) using hydraulic presses. In this spirit, carbon nanotubes can also be pressed into materials at high pressures to generate surface roughness.

18.2.5
Layer-by-Layer Deposition

Sequential deposition is a recurrent theme for the generation of patterned co-cultures for controlling cell–cell interactions. One variation on "sequential deposition" is to use thermally responsive polymers [40], whereby electron beam irradiation can be used to pattern a thin layer of thermally responsive acrylamide onto surfaces. Cells can then be cultured to confluence at 37 °C. The temperature is subsequently reduced, to induce the thermally responsive regions to become non-adhesive and force cell detachment. Once the detached cells have been removed, the temperature is increased to enable the adhesion of a second cell type on the exposed substrate. Layer-by-layer deposition of ionic polymers has also been used to generate patterned co-cultures. In this approach, a surface that is patterned with an ionic polymer is treated with an adhesive protein, resulting in preferential attachment of the protein to non-patterned regions, such that cell patterns can then be formed on the adhesive regions. Next, the non-adhesive surfaces can be treated with another layer of cell-adhesive ionic polymer. The subsequent deposition of a secondary cell type results in the formation of patterned co-cultures [86].

18.2.6
3D Printing

Traditionally, 3DP – a solid freeform fabrication technique – has been applied to orthopedic tissue engineering applications to generate channels and networks within scaffolds [61]. For ceramic scaffolds, printing relies on the ability to bond wetted regions of powdered base material, but generally a bed of powder may be printed with a particular binder solution which bonds the wetted regions of powder in the shape of the wetted regions into a monolithic mold. The subsequent removal of non-bonded powder produces the final structure. The technique may be performed sequentially in a layer-by-layer manner to produce complicated 3-D structures. Microsyringe deposition is similar to 3DP in that it relies on con-

trolled deposition, and the shape of the resulting structure is a direct function of deposition patterns. However, instead of rastering a printhead across the surface and depositing a ''glue'', the deposited material is typically a polymer which polymerizes to form the scaffold directly. Here, the resolution of the deposited lines of polymer is a function of a number of parameters such as syringe pressure and dimensions, as well as the solution viscosity. Complex structures are also generated through layer-by-layer application of this technique.

18.3 Outlook

The merger of novel biomaterials and nanofabrication strategies has led to dramatic enhancements in the complexity and biomimicry of today's tissue engineering constructs. Emerging tools for manipulating the micro- and nanoscale cellular environment have provided much insight into the fundamental biology of how cells interact with the surrounding components such as cell–cell, cell–soluble factors, and cell–ECM molecules. This knowledge can be used to direct cell fates, and can be incorporated into tissue engineering scaffolds. As our understanding of the relevant parameters increases, new nanomaterials and technologies that provide proper signals and environmental cues to cells provide exciting opportunities for the generation of clinically viable tissues. Significant challenges remain to be addressed, however, including the lack of suitable materials with the desired degradation rates, and the mechanical properties for the desired tissue. Another challenge is the optimization of scaffold architecture, including pore size, morphology, surface topography, and bioactivity. Also, new and optimized processing methods must be developed to address issues related to cell seeding, vascularization and scale up into 3-D structures. In addition, research is required to test and validate the *in-vivo* functionality of micro- and nanofabricated constructs, and to assess the performance of these constructs against existing clinically applied technologies.

References

1 Langer, R., Vacanti, J. P. (1993) Tissue engineering. *Science* **260**, 920–926.

2 Romanova, E. V., Oxley, S. P., Rubakhin, S. S., Bohn, P. W., Sweedler, J. V. (2006) Self-assembled monolayers of alkanethiols on gold modulate electrophysiological parameters and cellular morphology of cultured neurons. *Biomaterials* **27**, 1665–1669.

3 Lan, M. A., Gersbach, C. A., Michael, K. E., Keselowsky, B. G., Garcia, A. J. (2005) Myoblast proliferation and differentiation on fibronectin-coated self assembled monolayers presenting different surface chemistries. *Biomaterials* **26**, 4523–4531.

4 Hwang, J. J., Iyer, S. N., Li, L. S., Claussen, R., Harrington, D. A., Stupp, S. I. (2002) Self-assembling biomaterials: liquid crystal phases of

cholesteryl oligo(L-lactic acid) and their interactions with cells. *Proc. Natl. Acad. Sci. USA* **99**, 9662–9667.

5 Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., Ingber, D. E. (1997) Geometric control of cell life and death. *Science* **276**, 1425–1428.

6 Dertinger, S. K., Jiang, X., Li, Z., Murthy, V. N., Whitesides, G. M. (2002) Gradients of substrate-bound laminin orient axonal specification of neurons. *Proc. Natl. Acad. Sci. USA* **99**, 12542–12547.

7 Burdick, J. A., Khademhosseini, A., Langer, R. (2004) Fabrication of gradient hydrogels using a microfluidics/photopolymerization process. *Langmuir* **20**, 5153–5156.

8 Zaari, N., Rajagopalan, S. K., Kim, S. K., Engler, A. J., Wong, J. Y. (2004) Photopolymerization in microfluidic gradient generators: microscale control of substrate compliance to manipulate cell response. *Adv. Mater.* **16**, 2133–2137.

9 den Braber, E. T., de Ruijter, J. E., Ginsel, L. A., von Recum, A. F., Jansen, J. A. (1998) Orientation of ECM protein deposition, fibroblast cytoskeleton, and attachment complex components on silicone microgrooved surfaces. *J. Biomed. Mater. Res.* **40**, 291–300.

10 Walboomers, X. F., Croes, H. J., Ginsel, L. A., Jansen, J. A. (1999) Contact guidance of rat fibroblasts on various implant materials. *J. Biomed. Mater. Res.* **47**, 204–212.

11 van Kooten, T. G., Whitesides, J. F., von Recum, A. (1998) Influence of silicone (PDMS) surface texture on human skin fibroblast proliferation as determined by cell cycle analysis. *J. Biomed. Mater. Res.* **43**, 1–14.

12 Teixeira, A. I., Abrams, G. A., Bertics, P. J., Murphy, C. J., Nealey, P. F. (2003) Epithelial contact guidance on well-defined micro- and nano-structured substrates. *J. Cell Sci.* **116**, 1881–1892.

13 Sato, M., Webster, T. J. (2004) Nanobiotechnology: implications for the future of nanotechnology in orthopedic applications. *Expert Rev. Med. Devices* **1**, 105–114.

14 de Oliveira, P. T., Nanci, A. (2004) Nanotexturing of titanium-based surfaces upregulates expression of bone sialoprotein and osteopontin by cultured osteogenic cells. *Biomaterials* **25**, 403–413.

15 Price, R. L., Waid, M. C., Haberstroh, K. M., Webster, T. J. (2003) Selective bone cell adhesion on formulations containing carbon nanofibers. *Biomaterials* **24**, 1877–1887.

16 Webster, T. J., Siegel, R. W., Bizios, R. (1999) Osteoblast adhesion on nanophase ceramics. *Biomaterials* **20**, 1221–1227.

17 Webster, T. J. (2001) Nanophase ceramics: The future of orthopedic and dental implant material. In: Ying, J. Y. (Ed.), *Nanostructured Materials*. Academy Press, New York, pp. 126–166.

18 Webster, T. J., Ergun, C., Doremus, R. H., Siegel, R. W., Bizios, R. (2000) Enhanced functions of osteoblasts on nanophase ceramics. *Biomaterials* **21**, 1803–1810.

19 Webster, T. J., Ergun, C., Doremus, R. H., Siegel, R. W., Bizios, R. (2001) Enhanced osteoclast-like cell functions on nanophase ceramics. *Biomaterials* **22**, 1327–1333.

20 Stevens, M. M., George, J. H. (2005) Exploring and engineering the cell surface interface. *Science* **310**, 1135–1138.

21 Anderson, D. G., Levenberg, S., Langer, R. (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. *Nat. Biotechnol.* **22**, 863–866.

22 Anderson, D. G., Putnam, D., Lavik, E. B., Mahmood, T. A., Langer, R. (2005) Biomaterial microarrays: rapid, microscale screening of polymer-cell interaction. *Biomaterials* **26**, 4892–4897.

23 Flaim, C. J., Chien, S., Bhatia, S. N. (2005) An extracellular matrix microarray for probing cellular differentiation. *Nat. Methods* **2**, 119–125.

24 Thakar, R. G., Ho, F., Huang, N. F., Liepmann, D., Li, S. (2003) Regulation of vascular smooth muscle cells by micropatterning. *Biochem. Biophys. Res. Commun.* **307**, 883–890.

25 Miller, D. C., Thapa, A., Haberstroh, K. M., Webster, T. J. (2004) Endothelial and vascular smooth muscle cell function on poly(lactic-*co*-glycolic acid) with nano-structured surface features. *Biomaterials* **25**, 53–61.

26 Thapa, A., Webster, T. J., Haberstroh, K. M. (2003) Polymers with nano-dimensional surface features enhance bladder smooth muscle cell adhesion. *J. Biomed. Mater. Res. A* **67**, 1374–1383.

27 McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K., Chen, C. S. (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* **6**, 483–495.

28 Elbert, D. L., Hubbell, J. A. (2001) Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering. *Biomacromolecules* **2**, 430–441.

29 Schutt, M., Krupka, S. S., Milbradt, A. G., Deindl, S., Sinner, E. K., Oesterhelt, D., Renner, C., Moroder, L. (2003) Photocontrol of cell adhesion processes: model studies with cyclic azobenzene-RGD peptides. *Chem. Biol.* **10**, 487–490.

30 Okano, T., Yamada, N., Okuhara, M., Sakai, H., Sakurai, Y. (1995) Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. *Biomaterials* **16**, 297–303.

31 Lahann, J., Mitragotri, S., Tran, T. N., Kaido, H., Sundaram, J., Choi, I. S., Hoffer, S., Somorjai, G. A., Langer, R. (2003) A reversibly switching surface. *Science* **299**, 371–374.

32 Yeo, W. S., Yousaf, M. N., Mrksich, M. (2003) Dynamic interfaces between cells and surfaces: electroactive substrates that sequentially release and attach cells. *J. Am. Chem. Soc.* **125**, 14994–14995.

33 Zhu, X., Mills, K. L., Peters, P. R., Bahng, J. H., Liu, E. H., Shim, J., Naruse, K., Csete, M. E., Thouless, M. D., Takayama, S. (2005) Fabrication of reconfigurable protein matrices by cracking. *Nat. Mater.* **4**, 403–406.

34 Morin, O., Normand, C. (1986) Long-term maintenance of hepatocyte functional activity in co-culture: requirements for sinusoidal endothelial cells and dexamethasone. *J. Cell Physiol.* **129**, 103–110.

35 Fukuda, J., Okamura, K., Ishihara, K., Mizumoto, H., Nakazawa, K., Ijima, H., Kajiwara, T., Funatsu, K. (2005) Differentiation effects by the combination of spheroid formation and sodium butyrate treatment in human hepatoblastoma cell line (Hep G2): a possible cell source for hybrid artificial liver. *Cell Transplant.* **14**, 819–827.

36 Bhatia, S. N., Balis, U. J., Yarmush, M. L., Toner, M. (1999) Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J.* **13**, 1883–1900.

37 Bhatia, S. N., Balis, U. J., Yarmush, M. L., Toner, M. (1998) Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions. *Biotechnol. Prog.* **14**, 378–387.

38 Bhatia, S. N., Balis, U. J., Yarmush, M. L., Toner, M. (1998) Probing heterotypic cell interactions: hepatocyte function in micro-fabricated co-cultures. *J. Biomater. Sci. Polym. Ed.* **9**, 1137–1160.

39 Bhatia, S. N., Yarmush, M. L., Toner, M. (1997) Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts. *J. Biomed. Mater. Res.* **34**, 189–199.

40 Yamato, M., Konno, C., Utsumi, M., Kikuchi, A., Okano, T. (2002) Thermally responsive polymer-grafted surfaces facilitate patterned cell seeding and co-culture. *Biomaterials* **23**, 561–567.

41 Hirose, M., Yamato, M., Kwon, O. H., Harimoto, M., Kushida, A., Shimizu,

T., Kikuchi, A., Okano, T. (2000) Temperature-responsive surface for novel co-culture systems of hepatocytes with endothelial cells: 2-D patterned and double layered co-cultures. *Yonsei Med. J.* **41**, 803–813.

42 Khademhosseini, A., Suh, K. Y., Yang, J. M., Eng, G., Yeh, J., Levenberg, S., Langer, R. (2004) Layer-by-layer deposition of hyaluronic acid and poly-l-lysine for patterned cell co-cultures. *Biomaterials* **25**, 3583–3592.

43 Chiu, D. T., Jeon, N. L., Huang, S., Kane, R. S., Wargo, C. J., Choi, I. S., Ingber, D. E., Whitesides, G. M. (2000) Patterned deposition of cells and proteins onto surfaces by using three-dimensional microfluidic systems. *Proc. Natl. Acad. Sci. USA* **97**, 2408–2413.

44 Tang, M. D., Golden, A. P., Tien, J. (2003) Molding of three-dimensional microstructures of gels. *J. Am. Chem. Soc.* **125**, 12988–12989.

45 Stevens, M. M., Mayer, M., Anderson, D. G., Weibel, D. B., Whitesides, G. M., Langer, R. (2005) Direct patterning of mammalian cells onto porous tissue engineering substrates using agarose stamps. *Biomaterials* **26**, 7636–7641.

46 Khademhosseini, A., Yeh, J., Eng, G., Karp, J., Kaji, H., Borenstein, J., Farokhzad, O. C., Langer, R. (2005) Cell docking inside microwells within reversibly sealed microfluidic channels for fabricating multiphenotype cell arrays. *Lab on a Chip* **5**, 1380–1386.

47 Takayama, S., Ostuni, E., Qian, X. P., McDonald, J. C., Jiang, X. Y., LeDuc, P., Wu, M. H., Ingber, D. E., Whitesides, G. M. (2001) Topographical micropatterning of poly(dimethylsiloxane) using laminar flows of liquids in capillaries. *Adv. Mater.* **13**, 570–574.

48 Takayama, S., McDonald, J. C., Ostuni, E., Liang, M. N., Kenis, P. J. A., Ismagilov, R. F., Whitesides, G. M. (1999) Patterning cells and their environments using multiple laminar fluid flows in capillary networks. *Proc. Natl. Acad. Sci. USA* **96**, 5545–5548.

49 Takayama, S., Ostuni, E., LeDuc, P., Naruse, K., Ingber, D. E., Whitesides, G. M. (2001) Subcellular positioning of small molecules. *Nature* **411**, 1016.

50 Lucchetta, E. M., Lee, J. H., Fu, L. A., Patel, N. H., Ismagilov, R. F. (2005) Dynamics of *Drosophila* embryonic patterning network perturbed in space and time using microfluidics. *Nature* **434**, 1134–1138.

51 Jeon, N. L., Dertinger, S. K. W., Chiu, D. T., Choi, I. S., Stroock, A. D., Whitesides, G. M. (2000) Generation of solution and surface gradients using microfluidic systems. *Langmuir* **16**, 8311–8316.

52 Dertinger, S. K. W., Chiu, D. T., Jeon, N. L., Whitesides, G. M. (2001) Generation of gradients having complex shapes using microfluidic networks. *Anal. Chem.* **73**, 1240–1246.

53 Jeon, N. L., Baskaran, H., Dertinger, S. K. W., Whitesides, G. M., Van de Water, L., Toner, M. (2002) Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat. Biotechnol.* **20**, 826–830.

54 Chung, B. G., Flanagan, L. A., Rhee, S. W., Schwartz, P. H., Lee, A. P., Monuki, E. S., Jeon, N. L. (2005) Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. *Lab on a Chip* **5**, 401–406.

55 Dertinger, S. K. W., Jiang, X. Y., Li, Z. Y., Murthy, V. N., Whitesides, G. M. (2002) Gradients of substrate-bound laminin orient axonal specification of neurons. *Proc. Natl. Acad. Sci. USA* **99**, 12542–12547.

56 Taylor, A. M., Blurton-Jones, M., Rhee, S. W., Cribbs, D. H., Cotman, C. W., Jeon, N. L. (2005) A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nat. Methods* **2**, 599–605.

57 Cohen, S., Bano, M. C., Cima, L. G., Allcock, H. R., Vacanti, J. P., Vacanti, C. A., Langer, R. (1993) Design of synthetic polymeric structures for

cell transplantation and tissue engineering. *Clin. Mater.* **13**, 3–10.
58 Karp, J., Dalton, P., Shoichet, M. (2003) Scaffolds for tissue engineering. *MRS Bull.* **28**, 301–306.
59 Folkman, J., Hochberg, M. (1973) Self-regulation of growth in three dimensions. *J. Exp. Med.* **138**, 745–753.
60 Hutmacher, D. W., Sittinger, M., Risbud, M. V. (2004) Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. *Trends Biotechnol.* **22**, 354–362.
61 Seitz, H., Rieder, W., Irsen, S., Leukers, B., Tille, C. (2005) Three-dimensional printing of porous ceramic scaffolds for bone tissue engineering. *J. Biomed. Mater. Res. B Appl. Biomater.* **74**, 782–788.
62 Mironov, V., Boland, T., Trusk, T., Forgacs, G., Markwald, R. R. (2003) Organ printing: computer-aided jet-based 3D tissue engineering. *Trends Biotechnol.* **21**, 157–161.
63 Vozzi, G., Flaim, C., Ahluwalia, A., Bhatia, S. (2003) Fabrication of PLGA scaffolds using soft lithography and microsyringe deposition. *Biomaterials* **24**, 2533–2540.
64 Folch, A., Mezzour, S., During, M., Hurtado, O., Toner, M., Muller, R. (2000) Stacks of microfabricated structures as scaffolds for cell culture and tissue engineering. *Biomed. Microdevices* **2**, 207–214.
65 Yeong, W. Y., Chua, C. K., Leong, K. F., Chandrasekaran, M. (2004) Rapid prototyping in tissue engineering: challenges and potential. *Trends Biotechnol.* **22**, 643–652.
66 Bhatia, S. N., Chen, S. C. (1999) Tissue engineering at the micro-scale. *Biomed. Microdevices* **2**, 131–144.
67 Tsang, V. L., Bhatia, S. N. (2004) Three-dimensional tissue fabrication. *Adv. Drug Deliv. Rev.* **56**, 1635–1647.
68 Yoshimoto, H., Shin, Y. M., Terai, H., Vacanti, J. P. (2003) A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials* **24**, 2077–2082.
69 Fertala, A., Han, W. B., Ko, F. K. (2001) Mapping critical sites in collagen II for rational design of gene-engineered proteins for cell-supporting materials. *J. Biomed. Mater. Res.* **57**, 48–58.
70 Zong, X., Bien, H., Chung, C. Y., Yin, L., Fang, D., Hsiao, B. S., Chu, B., Entcheva, E. (2005) Electrospun fine-textured scaffolds for heart tissue constructs. *Biomaterials* **26**, 5330–5338.
71 Xu, C. Y., Inai, R., Kotaki, M., Ramakrishna, S. (2004) Aligned biodegradable nanofibrous structure: a potential scaffold for blood vessel engineering. *Biomaterials* **25**, 877–886.
72 Zhang, S. (2003) Fabrication of novel biomaterials through molecular self-assembly. *Nat. Biotechnol.* **21**, 1171–1178.
73 Hartgerink, J. D., Beniash, E., Stupp, S. I. (2001) Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science* **294**, 1684–1688.
74 Silva, G. A., Czeisler, C., Niece, K. L., Beniash, E., Harrington, D. A., Kessler, J. A., Stupp, S. I. (2004) Selective differentiation of neural progenitor cells by high-epitope density nanofibers. *Science* **303**, 1352–1355.
75 Whitesides, G. M., Ostuni, E., Takayama, S., Jiang, X. Y., Ingber, D. E. (2001) Soft lithography in biology and biochemistry. *Annu. Rev. Biomed. Eng.* **3**, 335–373.
76 Folch, A., Toner, M. (2000) Micro-engineering of cellular interactions. *Annu. Rev. Biomed. Eng.* **2**, 227–256.
77 Suh, K. Y., Seong, J., Khademhosseini, A., Laibinis, P. E., Langer, R. (2004) A simple soft lithographic route to fabrication of poly (ethylene glycol) microstructures for protein and cell patterning. *Biomaterials* **15**, 557–563.
78 Mrksich, M., Dike, L. E., Tien, J., Ingber, D. E., Whitesides, G. M. (1997) Using microcontact printing to pattern the attachment of mammalian cells to self-assembled monolayers of alkanethiolates on transparent films of gold and silver. *Exp. Cell. Res.* **235**, 305–313.

79 Koh, W. G., Revzin, A., Pishko, M. V. (2002) Poly(ethylene glycol) hydrogel microstructures encapsulating living cells. *Langmuir* **18**, 2459–2462.

80 Borenstein, J. T., Terai, H., King, K. R., Weinberg, E. J., Kaazempur-Mofrad, M. R., Vacanti, J. P. (2002) Microfabrication technology for vascularized tissue engineering. *Biomed. Microdevices* **4**, 167–175.

81 Kaihara, S., Borenstein, J., Koka, R., Lalan, S., Ochoa, E. R., Ravens, M., Pien, H., Cunningham, B., Vacanti, J. P. (2000) Silicon micromachining to tissue engineer branched vascular channels for liver fabrication. *Tissue Eng.* **6**, 105–117.

82 King, K., Wang, C., Kaazempur-Mofrad, M., Vacanti, J., Borenstein, J. (2004) Biodegradable microfluidics. *Adv. Mater.* **16**, 2007–2012.

83 Tan, W., Desai, T. A. (2003) Microfluidic patterning of cells in extracellular matrix biopolymers: effects of channel size, cell type, and matrix composition on pattern integrity. *Tissue Eng.* **9**, 255–267.

84 Tan, W., Desai, T. A. (2004) Layer-by-layer microfluidics for biomimetic three-dimensional structures. *Biomaterials* **25**, 1355–1364.

85 Tan, W., Desai, T. A. (2005) Microscale multilayer cocultures for biomimetic blood vessels. *J. Biomed. Mater. Res. A* **72**, 146–160.

86 Fukuda, J., Khademhosseini, A., Yeh, J., Eng, G., Cheng, J., Farokhzad, O. C., Langer, R. (2006) Micropatterned cell co-cultures using layer-by-layer deposition of extracellular matrix components. *Biomaterials* **27**, 1479–1486.