Norbert Pallua
Christoph V. Suschek
*Editors*

# Tissue Engineering

## From Lab to Clinic

Springer

Norbert Pallua • Christoph V. Suschek
Editors

# Tissue Engineering

## From Lab to Clinic

Springer

*Editors*
Univ.-Prof. Dr. med. Norbert Pallua
Universitätsklinikum, der RWTH Aachen
Klinik für Plastische Chirurgie,
Handund Verbrennungschirurgie
Pauwelsstr. 30
52074 Aachen
Germany
npallua@ukaachen.de

Prof. Dr. Christoph V. Suschek
Universitätsklinikum, der RWTH Aachen
Klinik für Plastische Chirurgie,
Handund Verbrennungschirurgie
Pauwelsstr. 30
52074 Aachen
Germany
csuschek@ukaachen.de

*Illustrator*
Piotr Gusta
6. Rue du Chemin Vert
94500 Champigny sur Marne, France

ISBN 978-3-642-02823-6 e-ISBN 978-3-642-02824-3
DOI 10.1007/978-3-642-02824-3
Springer Heidelberg Dordrecht London New York



*Cover design*: eStudioCalamar, Figueres/Berlin

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

# Preface

Although efforts to generate bioartificial tissues and organs for human therapies date back to the 80s, these efforts have only come closer to reality in the past 10 years. The possibility of such bioartifical tissues has been fueled by major advances in cell and molecular biology and the development of more sophisticated cell-culture technologies. The term *Tissue Engineering* has come to a broader application in the last five years and now encompasses the many interdisciplinary fields of knowledge that are crucial to generate or regenerate tissues or even whole organs. Tissue engineering has the potential to revolutionize health care, improving the treatment options and quality of life for millions of people worldwide, yet, saving enormous financial resources in terms of health care costs. One of its defining characteristics is that it draws upon and requires close collaboration among scientists in many diverse specialties. Cell and molecular biologists, biomaterials engineers, advanced imaging specialists, robotics engineers, and developers of equipment such as bioreactors, where tissues are grown and nurtured, are all part of the process of tissue engineering. Another characteristic of tissue engineering is that the field has brought together researchers worldwide in international collaborative efforts. Tissue engineering as a promising research is underway in all parts of the globe. The emergence of tissue engineering has coincided with the emergence of the Internet, making far-reaching collaborations more possible than ever before.

The topics in *Tissue Engineering: From Lab to Clinic*, addressed by world-renowned authorities, were selected to cover the spectrum from basic development to clinical application. Pertinent information on cell isolation and expansion, both in animals and humans, provides guidance for the clinical scientists interested in this area. Written by leading experts in the field, each chapter offers a detailed state-of-the-art overview of the respective field of research, the author's visions regarding his research area, as well as its limitations. This book provides a conceptual framework that includes the entire necessary background material in all areas of tissue engineering.

*Tissue Engineering: From Lab to Clinic* is intended to be a reference for first-year to senior-level graduate courses in Tissue Engineering, in departments of bioengineering, and for students who perform research in tissue replacement and restoration. Additionally, this book attempts to give guidance for students in biology, medicine, and life-sciences, working with primary and complex cell biology. This book, seeks to provide both undergraduate and graduate students with the scientific foundation of

tissue engineering. We therefore hope to provide a reference to biomedical engineering students, bioengineers, biological sciences graduate students, as well as their teachers, to managers and scientists in the biotech industry, and academic researchers.

Norbert Pallua
Christoph V. Suschek

# Contents

# Part I

# Basics and Principles of Tissue Engineering

# Micro- and Nanotechnology in Tissue Engineering

1

Daniela Coutinho, Pedro Costa, Nuno Neves, Manuela E. Gomes, and Rui L. Reis

## 1.1 Introduction

Tissue engineering (TE) is a rapidly growing scientific area [129] that aims to create, repair, and/or replace tissues and organs by using combinations of cells, biomaterials, and/or biologically active molecules [42, 119]. In this way, TE intends to help the body to produce a material that resembles as much as possible the body's own native tissue. By doing so, TE strategies promise to revolutionize current therapies and significantly improve the quality of life of millions of patients.

The classical TE strategy consists of isolating specific cells through a biopsy from a patient, growing them on a biomimetic scaffold under controlled culture conditions, delivering the resulting construct to the desired site in the patient's body, and directing the new tissue formation into the scaffold that can be degraded over time [42, 119].

Most of the presently existing TE techniques rely on the use of macrostructured porous scaffolds, which act as supports for the initial cell attachment and subsequent tissue formation, both in vitro and in vivo [88, 102, 113]. This kind of approach has been successful to a certain extent in producing relatively simple constructs relying on the intrinsic natural capability of cells and tissues to self-regenerate, remodel, and adapt. For this reason, cells have been the most significant factor in the generation of the tissue itself [33]. However, this natural capability of cells for adapting to its surrounding environment has limitations and that is the main reason why TE has not been able to generate complex thick tissues so far [47]. In fact, one of the most important drawbacks of the currently available constructs in TE approaches is related to the lack of means to generate effective oxygen and nutrient dispersion pathways that can reach a whole construct homogenously and, therefore, enable the functionality/viability of the construct upon implementation.

In order to generate constructs capable of accurately mimicking/replacing structures as defined and organized as complex tissues and organs, novel kinds of scaffolds and devices have lately been developed, which potentially allow obtaining a fine control over the cellular positioning, organization, and interactions [36]. For this, much has contributed the continuous technological development in the areas of micro- and nanotechnologies, both in terms of production methods and in analysis tools [107]. Developments in these areas may allow a finer control over the architecture of scaffolds, making them no longer simple substrates for cellular adhesion and proliferation, but most importantly, active agents in the process of tissue development [37]. Micropatterning integrated in a TE approach is a result of the combination of micro- and nanofabrication techniques with materials science and surface engineering, which results in a deep exploration of the microenvironment where cells are embedded [37, 90]. In TE, micro- and nanotechnologies can also be applied to fabricate biomimetic scaffolds with increased complexity to promote, for example, vascularization, also enabling to perform a series of high-throughput experiments (Fig. 1.1).

D. Coutinho, P. Costa, N. Neves, M.E. Gomes (✉), and R.L. Reis
3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal and PT Government Associated Laboratory, IBB – Institute for Biotechnology and Bioengineering, Braga, Portugal
e-mail: megomes@dep.uminho.pt

N. Pallua and C.V. Suschek (eds.), *Tissue Engineering*,
DOI: 10.1007/978-3-642-02824-3_1, © Springer-Verlag Berlin Heidelberg 2011

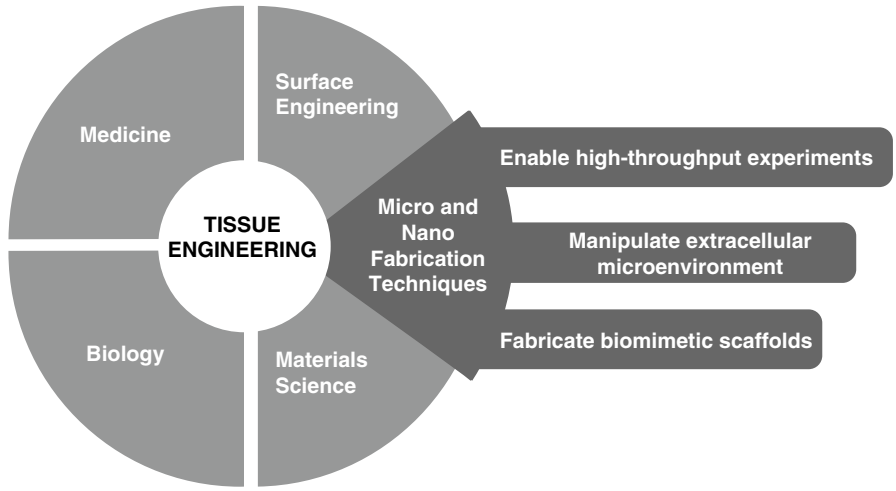



**Fig. 1.1** Schematic representation of the different areas of expertise involved in a tissue engineering (TE) strategy. Highlight to the inputs brought by micro- and nanofabrication techniques

The main aim of this chapter is to shed new light on the use of micro- and nanotechnologies in TE strategies, highlighting the need for new technologies to obtain an in-depth understanding of the microenvironment at the implantation site. This chapter also reviews some of the most widely used and most promising micro- and nanoscale technologies in TE strategies. Subsequently, the most advanced applications of micro- and nanofabrication techniques in different areas of TE will be described, showing their enormous potential for being applied to the improvement of the life quality of patients. Although the described techniques are usually applied for developing 2D structures, successful attempts to move towards 3D structures by means of micro- and nanofabrication techniques will be explored. Finally, the application of micro- and nanotechnologies in the development of cell/tissue culture systems (microbioreactors) will also be briefly discussed.

## 1.2 Aim of the Discipline

Micro- and nanotechnologies consist of the design, characterization, production, and application of structures, devices, and systems by controlling their shape and size at micrometric or nanometric scales. The properties of materials at atomic, molecular, and macromolecular scales are significantly different from those at a larger macroscopic scale, and for this reason, micro- and nanosciences have been receiving increasingly scientific, industrial, and social attention. The development of those areas may revolutionize and change the world as we know it and as we see it. Materials science and health are among the various areas that may mostly benefit from the evolution of micro- and nanotechnologies.

The major challenge encountered in biomaterials science is the issue of biocompatibility. Given its vital role, the control of the biocompatibility has become a major challenge and a main focus of research in the process of developing new materials for TE applications. Micro- and nanotechnologies, previously used in other research areas, are now integrated into the optimization of interactions occurring at the interface between materials and cells. By providing the possibility to tailor features such as micro- and nanotopographies, these novel technologies allow for the development of new micro- and nanostructured materials possessing unique properties allowing the development of solutions for the most complex end-applications.

Micro- and nanotechnologies can be regarded and designed from two different perspectives: the top–down and the bottom–up approach. As these terms suggest, the top–down approach can be pictured as large entities creating small devices while the bottom–up approach consists of small entities generating large devices. Both the perspectives are valid and interconnected and can lead to the development of new solutions for unaccomplished needs in several fields.

## 1.3 State of the Art

### 1.3.1 The Need for Micro and Nanotechnologies in Tissue Engineering Strategies

One of the major motivations for the increasing effort spent on designing and developing nanostructured materials for TE strategies is that natural tissues and the associated extracellular matrices are in fact composed of nanostructured materials [3, 69, 104] (Fig. 1.2). These highly organized and cooperative micro- and nanobuilding blocks assemble in a controlled way to ultimately build healthy tissues. When an implant first contacts its host environment, proteins are the first biological entities to interact with the surface of the biomaterial [10, 76]. In fact, the surface of the material gets covered by a layer of proteins, just a few seconds after implantation in a competitive process. Since cells do not interact with naked surfaces, the proteic layer adsorbed onto the material's surface will interact with the cell receptors at the cell membrane surface [110]. Thus, the presence of specific molecules is mandatory for a suitable attachment and proliferation of cells onto the surface of a given substrate [48]. Proteins are the key elements creating a bridge between the nonbiological surface of materials and cells [23]. Figure 1.3 represents, in a simplified way, the cascade of events occurring at a biomaterial implantation site. The adsorption of proteins onto the surface of biomaterials from a surrounding environment is a rather complex process. The adsorptive behavior of proteins is highly dependent on various surface properties such as chemistry, charge, topography, wettability, and surface energy [20, 89]. These parameters of the implant's surface will determine not only the amount of proteins adsorbed but also their type and conformation. The protein net charge and charge distribution, size, conformation, and hydrophobic domains are some of the characteristics of proteins that might have a profound effect on the implant surface properties and could ultimately influence the cell response to the implant's surface [10, 23, 76, 96]. Therefore, while adsorbing to surfaces, these nanoscale entities can either retain their original conformation or may have it altered in response to the environment conditions [23]. This surface-specific adjustment can result in the presentation of different aminoacidic regions to cells [23].

The initial contact between anchorage-dependent cells and the biomaterial's surface involves various biological molecules, such as extracellular matrix (ECM) proteins, cell membrane proteins, and cytoskeleton proteins [137]. Interactions between these biological molecules and their specific receptors induce signal transduction, and consequently influence cell growth and differentiation [40, 115]. Thus, the surface micro- and nanostructures are of huge importance as they will first dictate the pattern of protein adsorption, ultimately influencing the cell behavior through the motifs presented by those adsorbed proteins to the

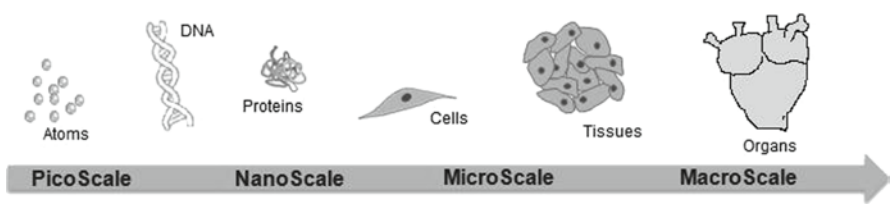



**Fig. 1.2** Nano- and Microscales in Nature


**Fig. 1.3** A glimpse at the cascade of events occurring at the implantation site

cells. Moreover, the cells themselves are also capable of directing their own position and orientation through a process called mechanosensing [112]. It has been shown that the cells can sense the topography of the surfaces to which they adhere and reposition themselves accordingly. Cells can respond both to the dimensions of the surface's roughness and also to its topography. Studies have revealed that cells can show a preference for adhering to surfaces within certain ranges of roughness, this factor being the determinant of the amount of cellular proliferation over that surface [6]. As for the surface's geometry, studies have shown that, when seeded in linearly grooved surfaces, cells tend to preferably adhere and grow longitudinally over the grooved lines [154]. The cell alignment is also favored in surfaces exhibiting grooves within certain width ranges [14, 31]. Figure 1.4 shows an example of a defined alignment of osteoblast-like cells (SaOs-2) on a patterned surface. Still, regarding geometry, studies have shown that cells tend to adhere preferably to randomly organized surfaces instead of geometrically organized ones. Thus, for the design of enhanced biomaterials to be used in TE strategies, it is of utmost importance to understand the cellular processes that lead to a more efficient tissue regeneration as well as those involved in the formation of new tissue avoiding triggering of undesired immunological or inflammatory responses. As referred, the interaction between polymeric biomaterials and biological elements is dictated by the processes occurring at the interface between these two phases. An approach to optimize the interaction of a biomaterial with its implantation site bioenvironment is by tailoring the properties of the material's surface with micro- and nanostructures that could elicit the desired behavior on the biological entities. For this, micro- and nanotechnologies can be used to overcome the limitations of the currently used techniques to fabricate biomaterials with effective micro- and nanosurface structures that could mimic as close as possible the micro- and nanobioenvironment.

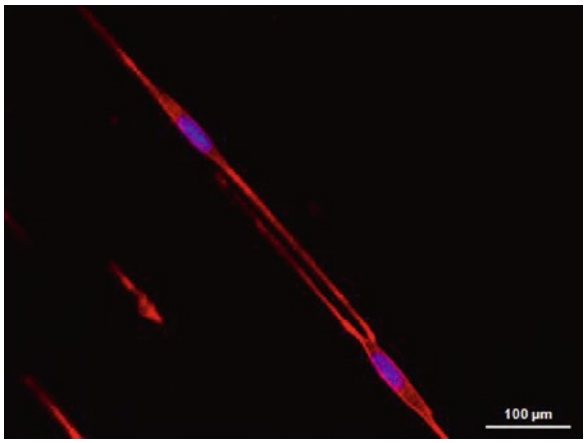



**Fig. 1.4** Cell alignment of osteoblast-like cells (SaOs-2) on a patterned surface with the cytoskeleton stained with phalloidin and the nucleus counterstained with DAPI

### 1.3.2 Micro and Nanofabrication Methods

Micro and nanofabrication is the general term used for describing the processes of fabricating miniature micro or nanoscaled structures by using macroscale devices. The earliest microfabricated devices consisted of semiconductor integrated circuits and were fabricated by simple surface treatments or methods such as lithography or chemical vapor deposition [45, 117]. Apart from integrated circuits, which are mainly two-dimensional, these technologies are commonly used in the production of devices such as microelectromechanical systems (MEMS) [27, 118], laser diodes [156], flat panel displays [100], or fuel cells [61].

Different strategies have been utilized to apply two-dimensional micro- and nanofeatured devices to TE. Microfluidic devices can be produced through various surface treatments or lithographic methodologies. These methodologies consist of creating patterns on the surface of materials, into which cells can be cultured and positioned into specific locations by physical constraints. This physical constraint can also be used for controlling the interaction of cells with its surroundings, as with other cells. The same grooves that are used for cellular constraint can also be used for selectively feeding cells by perfusing with cell culture medium using microfluidic systems [58].

As mentioned above, micro- and nanofabrication methods can be roughly divided in two areas following radically different concepts: bottom–up or top–down (Fig. 1.5). The latter normally includes photolithography, microcontact printing, microtransfer molding, capillary force lithography, scanning probe lithography (SPL), and electrospinning methods, which will be discussed in the following sections.

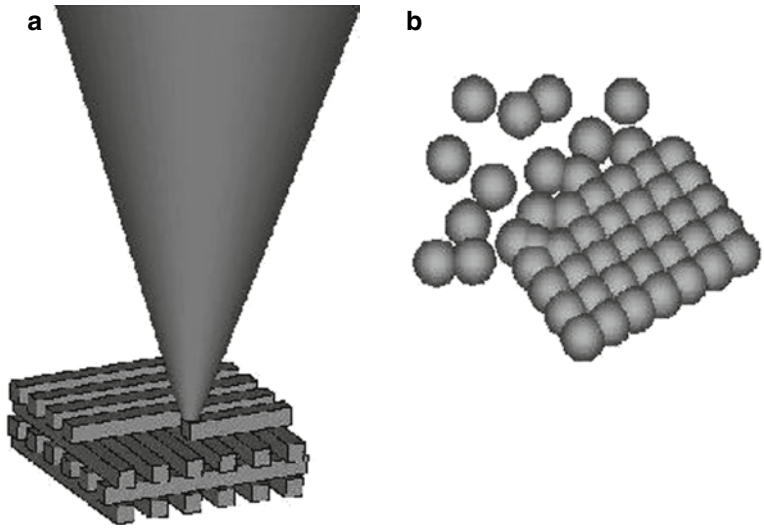



**Fig. 1.5** Schematic diagram of the (**a**) *top–down* and (**b**) *bottom–up* nanotechnology approaches for TE

Both approaches require a great know-how on the surface properties of materials. Besides having insights into the molecular events through thermodynamic and kinetic processes (bottom–up), researchers must also possess the skills to understand device miniaturization and fluidics, which are associated with top–down fabrication strategies. Interestingly, bottom–up and top–down approaches have in some cases merged to create systems that are more effective for tissue regeneration purposes. For example, microfluidic devices have been used to act as vasculature systems and test the interaction of targeted particles with the cells [133]. Also, 3D structures have been developed by combining bottom–up and top–down approaches. Hydrogels were micromachined by means of a top–down approach and then these patterned building blocks were assembled to form a more complex structure through a bottom–up approach [35, 147]. The next subsections are devoted to presenting an overview of the most used techniques of each approach.

#### 1.3.2.1 Bottom–Up Approach

The bottom–up approaches mainly rely on the natural assembly of atoms, molecules, or other nanosized building blocks such as peptide residues (dimensions typically of 2–10 nm [83]). Nature has already perfectly controlled micro- and nanoscale components by macromolecular recognition of various biological materials. Therefore, scientists are maintaining the trend of learning from nature how to design materials and systems using similar processes as the ones observed in nature [70]. The molecular assembly of building blocks by controlled reactions makes this technique more economic as compared to the conventional top–down methods [83].

Indeed, biologically inspired self-assembly holds great promise in the development of nanostructures [150]. For instance, research has shown that amphiphilic peptides can self-assemble to form hydrogels with great potential for TE strategies [150]. Moreover, self-assembled scaffolds functionalized with specific peptide moieties can be produced by incorporating these functional groups into the initial building molecule.

Of great interest are also the self-assembled-monolayers (SAMs), well established for the study of surface modification [114]. The SAMs are produced when a substance spontaneously forms a molecular monolayer on a surface [43]. Work reported by Shuguang Zhang and collaborators [151, 152] has shown that by observing the processes by which supramolecular structures are assembled in nature, the synthesis of novel synthetic materials can be developed. Thus, they have been able not only to coat surfaces by molecular assembly but also to develop nanofiber peptide and protein scaffolds with approximately 10 nm in diameter [150], which have proven to be suitable for applied studies in TE. Likewise, Frank Gu and collaborators [73] have successfully developed polymeric nanoparticles, which were formulated by the self-assembly of an amphiphilic triblock copolymer composed of end-to-

end linkage of poly(lactic-co-glycolic acid) (PLGA), polyethyleneglycol (PEG), and the A10 aptamer (Apt).

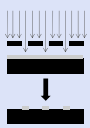
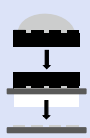
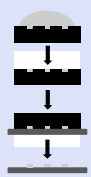
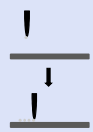
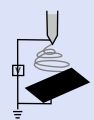
#### 1.3.2.2 Top–Down Approach

Most of the techniques used in the development of micro- and nanopatterned surfaces for TE purposes follow this route. Frequently, their origin is in other fields of research such as microelectronics fabrication. Nevertheless, all of these techniques are based on the development of micro- and nanofeatures on the surface of a material by means of larger tools. In the next sections, we present an overview of the most relevant techniques used in micropatterning biological materials, as well as their advantages and limitations for this purpose. Photolithographic techniques are widely developed and optimized for patterning cells. Nonetheless, disadvantages of this technique, mainly regarding biocompatibility issues, have highlighted the interest on alternative techniques based on soft-lithographic methods, such as microcontact printing, microtransfer molding and molding in capillaries. Likewise, scanning probe lithographic methods based on atomic force microscopy (AFM) are emerging as useful techniques for cell patterning applications. Additionally, to these techniques, the development of micro- and nanopatterned surfaces by means of electrospinning method has demonstrated a notable importance in TE strategies, demonstrated by the exponentially increasing number of publications on this subject over the years. Tables 1.1 and 1.2 present a summary of the technologies described in the next sections, concerning its resolution, materials that can be used, as well as their most advantageous and disadvantageous features.

##### Photolithography

Photolithography has been one of the first techniques to be applied in the micropatterning of surfaces [135] for various areas including for the biomedical field.

**Table 1.1** Summary of the nano- and microtechnologies most used

| | Photolithography | Soft lithography | | | | Electrospinning |
|---|---|---|---|---|---|---|
| | | Microcontact printing | Microtransfer molding | Molding in capillaries | Scanning probe lithography | |
| *Resolution* | 37 nm | 35 nm–1 μm | 250 nm | 50 μm | 20–30 nm | 5 nm–1 μm |
| *Scheme of the technique* |  |  |  |  |  |  |
| *Most advantageous feature(s)* | Precise control of the features | Simplicity<br>Flexibility<br>Allows for multipattern<br>Applied to nonplanar surfaces | Applied to nonplanar surfaces<br>Allows to generate 3D structures<br>Pattern large areas | Allows to produce different patterns in a parallel fashion<br>The use of wet patterns – broaden applications<br>Creation of gradients | Precise control of the added groups | Structures similar to ECM<br>High range of applicable materials |
| *Most disadvantageous feature(s)* | Polymers added with photosensitive compounds | Structural constraints of the stamp<br>Low control of the ligand density | Microstructures have a thin film between the raised features | Patterned geometries limiting | Limited to a low range of patterned sizes | Poor mechanical properties |
| *Reference* | [41] | [64, 93] | [153] | [93] | [16] | [71] |

Briefly, photolithography involves the placement of a precursor polymer solution and a UV light photoinitiator onto the substrate of choice, so that it forms a thin but uniform film (~1 μm thick) [1]. Photopolymerization is further performed under ultraviolet (UV) light using a mask to define selected exposure areas. Only the exposed regions of the prepolymer, which are a result of a patterned mask that is placed in between the light source and the precursor polymer solution, are submitted to photopolymerization. The surface is washed, leaving the patterned projections. Even though there are several variations of photolithographic methods, all rely on the use of a photoresistant mask with the desired pattern and the exposure of a precursor polymer solution to UV radiation.

Despite the enormous potential offered by this technology, the application of photolithography to patterning in biomedical applications presents some drawbacks, such as: (1) the high costs associated with the necessary equipment, namely, those related with the fabrication of the photoresistant mask, (2) the need for clean-room facilities, (3) the extra expertise required from the biologists, and (4) the use of chemical compounds which may be toxic to cells and induce denaturation of proteins.

## Soft Lithography

As an attempt to overcome the limitations of photolithograpic methods, a set of techniques entitled soft-lithographic methods have been developed by Whitesides and his research group [93]. Like photolithography, these techniques also make use of a micropatterned surface to generate the pattern of interest in the desired substrate. In this case, an elastomeric mold is used to transfer the patterns into the surfaces, determining the term "soft." In soft lithography, a stamp is created by molding an elastomer, which is usually produced using polydimethylsiloxane (PDMS). It offers several advantages including being biocompatible and permeable to gases. Soft-lithographic methods mainly comprise microcontact printing, microtransfer molding, and molding in capillaries, also known as capillary force lithography.

The general advantages of the aforementioned methods comprise : (1) the lower costs of softer materials, (2) their potential biocompatibility, (3) PDMS surface can be replicated repeatedly from the microfabricated master, and (4) the biopatterning does not damage the stamp surface.

### *Microcontact Printing*

Microcontact printing (μCP) was pioneered by Whitesides and his group in 1994 [136] and allows for patterning molecules onto a surface, resulting in control over the protein adsorption and cell behavior. Briefly, in this method, a stamp is produced by replica molding, which is usually made with PDMS. It involves inking the stamp with the substance to be transferred to the substrate. As the solvent evaporates, the molecules to be printed become deposited, and by removing the stamp from the surface, a pattern is created [37]. The first major application of this contact transfer-based method in the biomedical field consisted of the stamping of alkanethiolate inks, resulting in the formation of self-assembled alkythiol monolayers [87, 136]. Nevertheless, efforts have been made toward the use of different ink solutions [106] to pattern polymers [8, 132], DNA [67, 124], proteins [13, 32, 97, 105], or even cells [87, 116, 125]. However, the interactions between the stamp and the ink solution need to be controlled since PDMS is hydrophobic (Table 1.1).

The stamp being deformable allows detachment from the substrate without smearing the inked pattern. Moreover, because of its additive nature, it can be applied to nonplanar surfaces and develop multiprotein patterns [13]. Also, μCP is convenient for printing large areas (100 $cm^2$) in a single stamping step. Once the stamp is available, multiple copies of the pattern can be produced. Even so, it possesses some inherent disadvantages: (1) the density of the stamped material is not easily controlled [37, 106], (2) the elastomeric stamp might swell leading to a change on the pattern geometry [37], and also (3) contamination of the patterns with unpolymerized low molecular weight siloxane from the elastomeric stamp my compromise the process [101, 141].

## Microtransfer Molding

Microtransfer molding (μTM) was described for the first time in 1996 [153] and is a technique which allows for generating rather complex 3D structures. In μTM, an elastomeric mold usually made of PDMS

**Table 1.2** Examples of controlling cell microenvironment using micro- and nanotechnologies

| Application | Micro-/ nanotechnology | Cell–microenvironment interaction | Material | Features | Cells | Effect observed | Reference |
|---|---|---|---|---|---|---|---|
| *Bone* | Photolithography | Cell orientation and behavior | – | 330 nm deep and either 10, 25, or 100 μm in width | HOBs | Groove/ridge topographies are important modulators of both cellular adhesion and osteospecific function, and, critically, groove/ridge width is important in determining cellular response | [17] |
| | Photolithography | Cell behavior | Photocrosslinkable chitosan | 500–500 or 100–100 μm | SaOs-2 | The method utilized allowed to create patterned cell cocultures by using lysozyme or layer-by-layer surface switching | [51] |
| | Photolithography combined with electrochemical micromachining | Cell shape and behavior | Titanium | Cavities with 100, 30, and 10 μm diameters | MG63 osteoblast-like cells | Cell attachment depends on cavity spacing, cell growth and aggregation depends on cavity dimensions, and cell morphology depends on the presence of submicron-scale structural features | [155] |
| | Soft lithography | Cell orientation and behavior | Collagen | Groove Width: 27 mm; groove depth: 12 mm; ridge width: 2 mm | Mesenchymal osteoprogenitor cells | Cell alignment can be significantly affected by the topography of the carrier surface; appropriate surface topography can enhance bone formation | [12] |
| | Microcontact printing | Cell behavior | PHBV | 1–10 μm groove width; 5–30 μm groove depth | Rat mesenchymal stem cell-derived osteoblasts | Microtopographies on PHBV can improve osseointegration when combined with chemical cues; microgrooves and cell adhesive-protein lines on PHBV can guide selective osteoblast adhesion and alignment | [53] |
| | Microcontact printing combined with hot-embossing imprint lithography | Cell orientation and behavior | Polyimide; fibronectin | 8 μm wide grooves 4 μm deep (hot-embossing printing); 10 μm wide (microcontact printing) | MC3T3-E1 osteoblast-like cells | Although chemical patterns induced stronger alignment than mechanical topography when presented separately, mechanical topography dominated the alignment for all the chemical patterns when combined | [25] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Electrospinning | Cell orientation and behavior | SPCL | Diameter of fiber 400 nm (average) | SaOs-2 | The presence of the nanofibers induced the cell morphology to change into a more stretched and spread shape, increased cell activity, and viability | [127] |
| | Electrospinning | Cell orientation | PCL | Diameters of the fibers ranging between 200 nm and 1.2 μm | SaOs-2 | The cells integrate with the surrounding fibers and migrate into the inner nanofibrous structure to form a three-dimensional cellular network | [78] |
| *Cartilage* | Replica molding | Controlled microenvironment | PDMS; glass coverslip | Channels 300 μm wide and 100 μm high | Chondrocytes isolated from articular cartilage | The method associated with an inverted microscope allowed to monitor real-time cell size changes; articular chondrocytes exhibited a trend of increasing changes in cell size with decreasing osmotic loading frequency | [24] |
| | Photolithography | Cell behavior | HA on PET | Channels 25 and 5 μm wide and with the same spacing | Articular knee chondrocyte | Micropatterned HA induced adhesion, migration, alignment, and differentiation of chondrocytes | [11] |
| | Photolithography | Cell shape and behavior | Agarose gel | 15–65 μm wide; 40 μm deep | Chondrocytes isolated from avian sterna | Micropatterned polymer gels were subsequently applied as scaffolds for chondrocyte culture and proved effective in maintaining the key aspects of the chondrogenic phenotype (rounded cell morphology; production of type II collagen) | [94] |
| *Vascular* | Soft lithography | Controlled microenvironment | PDMS | 10 μm wide, 3 μm deep, 10 μm distance between each groove | MSCs | Microgrooved patterns induced global gene expression changes (an increase in the smooth muscle marker calponin one, a decrease in cartilage matrix markers, and alterations in cell signaling) | [66] |
| | Soft lithography | Cell shape and behavior | PDMS and collagen | Groove and ridge widths of 330 nm each; depth of 100 nm | VSMCs | Nanopatterns could successfully guide VSMCs along the pattern axes, improving collagen construct strength | [44] |
| | Soft lithography | Cell behavior under shear stress | PU | 95 μm wide and 32 μm deep | Endothelial cells | Endothelial cell retention is improved on micropatterned surfaces, which could help in reducing the thrombogenicity of implanted grafts | [34] |

(*continued*)

**Table 1.2** (Contiuned)

| Application | Micro-/ nanotechnology | Cell–microenvironment interaction | Material | Features | Cells | Effect observed | Reference |
|---|---|---|---|---|---|---|---|
| | Soft lithography associated with melt molding and particulate leaching | Cell shape and behavior | PCL and PLGA | 48 μm grooves; 5 μm deep; 12 μm spacing | VSMCs | VSMCs seeded into PLGA leached micropatterned PCL scaffolds maintain similar degrees of alignment as seen on nonporous micropatterned scaffolds (findings associated with promoting in vivo-like VSMC morphology, enhanced ECM production, and decreased proliferation) | [109] |
| | Microfluidic patterning | Cell shape and behavior | HA hydrogels | | Cardiomyocytes | The linearly aligned myocytes detached from the surface and formed contractile cardiac organoids | [55] |
| | Microfluidic patterning | Cell-ligands | PEG | Gradient generation of tethered RGDS through microfluidics/photopolymerization process | HUVEC | The adhesive ligand gradients modulated the spatial distribution of attached endothelial cells | [57] |
| | Microfluidic patterning | Cell-ligands | PDMS | Width 50–1,000 μm and spacing between them 50–100 μm; patterned adhesion molecules: P-selectin, E-selectin, von Willebrand factor | Neutrophils, ChineseHamster ovary cells (CHO), platelets | The adhesion and rolling of three different cell types was controlled by patterning microfluidic channels with different ligands | [91] |
| | Electrospinning | Cell orientation and behavior | P(LLA-CL) | Diameters of the fibers ranging from 200 to 800 nm | SMCs | The SMCs attached and migrated along the axis of the aligned nanofibers and expressed a spindle-like contractile phenotype | [144] |
| | Microcontact printing | Cell behavior | PDMS | Width of 20 and 115 μm | Bovine aortic endothelial cells | The presented cell morphology may interfere with the mechanisms sensing the physical cues, therefore responding differently to shear stress | [74] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Neural* | Microcontact printing | Cell–cell; Cell behavior | Silicon oxide | 3–20 μm in line width; 3.5 nm high of the layer | Rat hippocampal neurons | The immobilized neurons showed resting membrane potentials comparable with controls and were capable of eliciting action potentials after 1 day of culture | [111] |
| | Microcontact printing | Cell orientation and behavior | Polystyrene; PDMS | 4–6 μm wide lines | Rat embryonic cortical neurons | Cells comply well with the pattern and form synaptic connections along the experimentally defined pathways | [130] |
| | Replica molding | Cell shape and behavior | Poly(glycerol–sebacate) on sucrose-coated microfabricated silicon | 2–5 μm in wavelength; depth of 0.45 μm | Bovine aortic endothelial cells | Cells cultured on substrates with smaller pitches exhibited a substantially higher frequency of cell alignment and smaller circularity index; this system showed to be appropriate for in vivo applications | [14] |
| | Electrospinning | Cell orientation and behavior | PLLA | Average diameter of 300 nm (concentration of 2% of PLLA) and 1.5 μm (concentration of 5% of PLLA) | NSCs | The rate of NSC differentiation was higher for PLLA nanofibers than that of microfibers and it was independent of the fiber alignment | [145] |
| | Electrospinning | Cell orientation and behavior | PCL | Fiber diameter around 250 nm | hESCs | When EBs were cultured onto PCL nanofibers, they were capable of differentiating into mature neural lineage cells including neurons, oligodendrocytes, and astrocytes. The aligned nanofibers could also direct the neurite outgrowth | [142] |
| *Liver* | Photolithography | Cell–cell (3D structure) | PEG hydrogels | 500 μm in width | Hepatocytes | Three-dimensional photopatterned constructs were cultured in a continuous flow bioreactor for 12 days where they performed favorably in comparison to unpatterned, unperfused constructs | [126] |
| | Photolithography | Controlled microenvironment | PDMS | Through-holes (500 μm with 1,200-μm center-to-center spacing) | Primary rat hepatocytes; primary human hepatocytes; 3T3-J2 fibroblasts | The miniaturized, multiwall culture system for human liver cells with optimized microscale architecture was able to maintain phenotypic functions for several weeks | [59] |

(*continued*)

**Table 1.2** (Contiuned)

| Application | Micro-/ nanotechnology | Cell–microenvironment interaction | Material | Features | Cells | Effect observed | Reference |
|---|---|---|---|---|---|---|---|
| | Photolithography combined with robotic microarraying of proteins | Controlled microenvironment | PEG | Collagen spots with 170 μm in diameter; 30–30 μm PEG wells | Rat hepatocytes | When exposed to micropatterned surfaces, hepatocytes interacted exclusively with collagen-modified regions, attaching and becoming confined at a single-cell level within the hydrogel wells | [2] |
| | Photolithography combined with anisotropic wet etching | Controlled microenvironment | Titania ceramic | Circles 200 μm in diameter; heights ranging from 50 to 135 μm | Rat liver hepatocytes | The cells mainly aggregate within the cavities, and only single cells or small clusters are attached to the rims between the pits | [95] |
| | Soft lithography | Controlled microenvironment | PDMS; polycarbonate | Branch pattern; main inlet and outlet channels are 2,650 μm; the channels progressively branch and decrease to 35 μm in width to form the smallest structures | Hepatoma cell line HepG2/C3A; primary liver cells | The design provides sufficient oxygen and nutrient mass transfer to support the viability and function of both a highly metabolic hepatoma cell line and primary rat hepatocytes | [22] |
| | Electrospinning | Cell–cell | PCLEEP | Diameters of the fibers ranging from 300 nm to 1.5 μm | Primary rat hepatocytes | Galactosylated PCLEEP nanofiber mesh exhibits the unique property of promoting hepatocyte aggregates within the mesh and around the fibers, forming an integrated spheroid–nanofiber construct | [29] |
| Stem cells | Photolithography | Cell orientation | Silicon | 5 μm in width; 5 μm in spacing; 1.6 μm in depth | MSCs | The cells were shown to inhabit the grooves rather than ridges and exhibited an elongated shape, with unusually long processes | [149] |
| | Photolithography | Cell behavior | PLLA | 3 μm in width; 100 μm in spacing; 1.5 μm in depth | Mouse bone marrow stromal precursors | The microtopography showed to influence the differentiation of cells to adipocytes, also affecting the rate of lipid production | [26] |

| | | | | | | |
|---|---|---|---|---|---|---|
| Soft lithography | Cell shape and behavior | CCG blended membranes | 200 μm in width and 80 μm in depth | hMSCs | Some of the cells formed a bridge-like structure between two adjacent ridges; the micropatterned CCG membranes could be used to regulate the distribution, alignment, proliferation, and morphology of hMSCs during cell culture in vitro | [148] |
| Soft lithography | Cell differentiation; controlled microenvironment | Cell-repellant PEG | 150–40 μm diameter microwells | hESCs | The microwell technique could be a useful approach for in vitro studies involving ES cells and, more specifically, for initiating the differentiation of EBs of greater uniformity based on controlled microenvironments | [50] |
| Soft lithography | Cell-ECM | PDMS | 3.5 mm in diameter bioreactors, arranged in a 4×3 array | hESCs | The microbioreactor array that combines the advantages of microarrays with those of bioreactors, provided means to study the growth and differentiation of hESCs under controlled conditions | [21] |
| Soft lithography | Controlled microenvironment | PDMS | Cylinders 200 μm in diameter and 120 μm in height | hESCs; MEFs | While exhibiting a similar viability and self-renewal profile as that of hES cells grown on flat surfaces, hES cells grown on microwell-patterned substrates show a greater level of homogeneity in aggregate size | [56] |
| Electrospinning | Controlled microenvironment | Blend of poly(DL-lactide-co-glycolide) and collagen I | Diameter of fibers ranging from 100 to 500 nm in diameter | BM-HSCs | Besides acting as an in vitro HSC culture system for supporting hematopoiesis , the constructs may also act as an efficient captor and carrier for HSCs | [75] |
| Electrospinning | Controlled microenvironment | PES | Fibers with an average diameter of 529 ± 14 nm | HSPCs | Aminated nanofiber mesh could further enhance the HSPC-substrate adhesion and expansion of CFU–GEMM forming progenitor cells | [28] |

*Legend*: primary human osteoblasts (HOBs); Human osteogenic sarcoma cells (SAOS-2 cells); poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV); starch/polycaprolactone (SPCL); polycaprolactone (PCL); polydimethylsiloxane (PDMS); hyaluronic acid (HA); polyethylene-terephthalate (PET); mesenchymal stem cells (MSCs); bovine aorta cells (VSMCs); polyurethane (PU); polylactic-co-glycolic acid (PLGA); poly(ethylene glycol) (PEG); human umbilical vein endothelial cells (HUVEC); poly(L-lactid-co-ε-caprolactone) P(LLA-CL); human coronary artery smooth muscle cells (SMCs); poly(L-lactic acid) (PLLA); neural stem cells (NSCs); human embryonic stem (hESCs) cells; poly(e-caprolactone-co-ethyl ethylene phosphate) (PCLEEP); chitosan–collagen–gelatin (CCG); Human mesenchymal stem cells (hMSCs); murine embryonic fibroblasts (MEFs); hematopoietic stem cells (BM-HSCs); polyethersulfone (PES); hematopoietic stem/progenitor cells (HSPCs)

is produced by replica molding with the pattern of interest. A drop of the prepolymer solution is poured onto the mold and the excess gently removed [141]. The filled mold is placed in contact with the substrate, and the prepolymer solution is cured either by thermal or photochemical processes. After the procedure, the mold is peeled off leaving the pattern on the polymer. By stacking layers, 3D structures can be produced by means of μTM. Moreover, it presents some other useful characteristics [141, 153]: (1) it allows for patterning nonplanar surfaces, (2) it allows for fabricating structures in large surface areas (3 $cm^2$) [141] in a short period of time, (3) it is possible to rapidly replicate microstructures from the mold, (4) two or more components can be used to uniformly create microstructures, and (5) it is applicable to a wide range of materials. Depending on the applications, the main shortcoming of this technique is due to the thin films that are formed between the raised features [141].

#### Molding in Capillaries (Capillary Force Lithography)

Microfluidic patterning using capillaries, also known as capillary force lithography, allows for patterning a surface of a material from the flow of a solution [120–122]. Microchannels (or capillaries) are formed by the void spaces when the PDMS structure is placed on top of the substrate. Thus, in opposition to the aforementioned techniques, the material is added to the surface in the areas where the stamp is not in contact with the surface. Through capillary forces, these microchannels deliver the targeted fluid to the restricted areas of the substrate, allowing for a selective microfluidic patterning. The typical channel size is 50 μm, at which the fluids have a laminar flow with Reynolds Number ranging from 0.1 to 1 [93]. Therefore, if two streams coming from different inlets are to flow in a common channel, they will flow in parallel without turbulent mixing, and with diffusion only among both the fluids at the interface. This characteristic makes molding in capillaries a really interesting method for developing gradients. Lately, a great emphasis has been laid toward the generation of gradients by using microchannels [57]. Nevertheless, this technique does not simply allow to be used as a vehicle to deposit compounds, but also as a manner to cure itself into the surface or to remove a material.

Microfluidic patterning is very useful since it allows for selective protein immobilization and selective cell adhesion. Moreover, if gradient systems are used, it is possible to analyze the influence that two or more different drugs or other different compounds have on a single cell. Also, capillary force lithography is often used for immunoassays. The small volume of reagents required by this technique makes it a very powerful and unique tool for screening tests. However, the patterns are limited to the channel geometries [37].

#### Scanning Probe Lithography

Scanning Probe Lithography (SPL) embraces a range of techniques that involve the surface modification at the nanoscale by means of scanning probes such as AFM and scanning tunneling microscopes (STM) [41, 86]. By utilizing AFM and STM tips, SPL techniques enable to precisely pattern atoms or clusters of atoms onto a surface [41]. There are mainly three SPL subareas. The first one is entitled dip-pen nanolithography and allows for creating patterns of 15 nm. In this process, an AFM tip is inked with the material to be patterned, and while scanning a substrate, the tip transfers the material. For this, the humidity present plays an important role. Another area of SPL consists of the selective removal of a material from a surface. This is achieved by applying high pressure with the AFM tip onto the coated surface. Usually, the molecules that are removed from the surfaces are SAMs. Finally, the third area is based on the localized chemical modification of the surface, either by electrochemical anodization or by using a conductive AFM or STM tip.

### 1.3.2.3 Electrospinning

Electrospinning is a well-known and ubiquitous technique that enables the production of nanofibers and has been used by many researchers to make nanofibrous matrices for TE applications [46, 60, 71, 77, 79, 80, 84, 123]. The process of electrospinning results from the application of a high voltage electrostatic field operated between a metallic capillary of a syringe and a grounded collector. As a result of this electrostatic field, a solution drop deposited at the tip of the capillary tube elongates into long and thin fibers and projects over the grounded collector. The

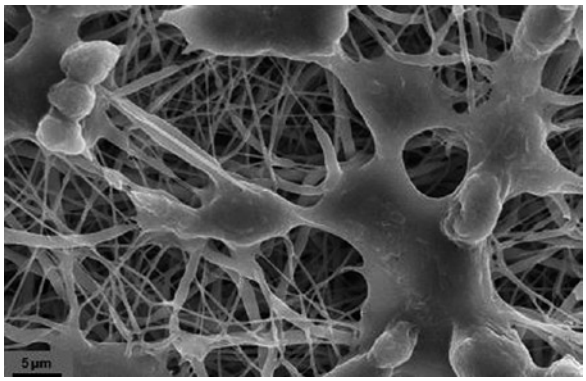



**Fig. 1.6** Nanofiber mesh of polycaprolactone obtained by electrospinning cultured with SaOs-2

nanofibers produced by electrospinning are collected into a nonwoven web, which generally originate a random fiber orientation mesh with limited mechanical properties.

Scaffolds produced by this technique were shown to closely mimic the structure and morphology of native ECM that consists of fibrilar structures with nanosized diameters [68, 103, 143].

This technique also provides the possibility of producing nanofibrous scaffolds from native polymers such as collagen and elastin. TE constructs based on these materials produced by electrospinning have been widely reported [18, 80, 81], once they have been able to direct cell alignment and support cell growth (Fig. 1.6).

## 1.4 Clinical Applications

Clinical applications of the constructs developed by the synergies between the TE and the micro- and nanofabrication fields are still ahead of us. Even, the clinical applications of the products obtained using TE strategies are still limited to a very small number of approved products, such as collagen membranes for cartilage repair. Nevertheless, there are already some microfabricated devices on the market aimed for tissue regeneration. An example is MotifMesh™, obtained by the combination of micromachined layers of a biocompatible polymer that allow creating a controlled ECM.

Interestingly, Johnson et al. [49] stated that the strategic directions in TE leading to successful clinical applications would involve a closer interface between TE and micro- and nanotechnologies by enhancing cell biology and biochemistry. It is expected that the intense research effort focused on these technologies will produce significant contributions to overcome some known bottlenecks in TE. Inputs would be needed in the following areas: (1) cell behavior in different culturing conditions, (2) cell–cell interactions in cocultures, usually achieved by means of patterned surfaces, (3) cell-ECM communication by high-throughput platforms, or (4) the influence of several soluble factors on specific cells through microfluidic systems (Fig. 1.7). Although much work is still required, the ability to design and manipulate surface properties at the micro- and nanoscale has already shown to be useful for different applications in TE. However, most of the micro- and nanotechnologies proposed for TE applications have been focused on 2D structures. Besides the potential shown at a 2D level, micro- and nanotechnologies should also be extended and exploited to produce 3D structures. Support systems for tailoring cell behavior and for tissue regeneration developed by those technologies have shown positive research results; currently, efforts are directed toward mimicking the natural environment in tissues with increasing accuracy. Presently, microbioreactor systems are being developed that will enable tissue growth in a tightly controlled microenvironment. The next sections highlight the opportunities for biomaterials development offered by micro- and nanotechnologies with proteins and, subsequently, the

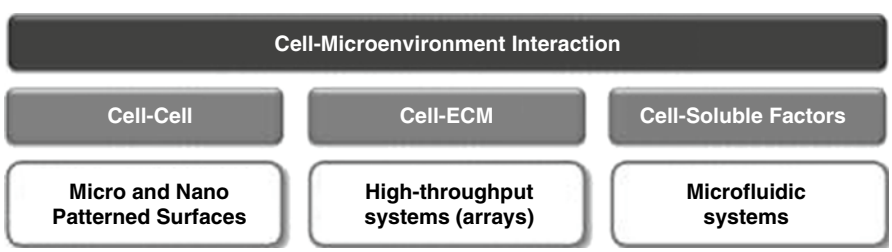



**Fig. 1.7** Schematic representation of the micro- and nanoplatforms used for TE applications

cells aiming the regeneration of the above-mentioned tissues, both in 2D and 3D. An overview of the more recent and promising advances in the development of microbioreactors will also be presented.

### 1.4.1 Micro and Nanotechnologies in the Development of Enhanced Constructs for Tissue Engineering

In the development of constructs for TE strategy by means of micro- and nanotechnologies, researchers must pay attention to two important parameters: the design of the patterns should mimic as much as possible the native structural environment; and the biomaterial selected to be the groundwork of the patterned system should have appropriate characteristics for a specific application. During the last couple of decades, great efforts have been directed toward creating systems that could resemble the native environment of tissues. In fact, important areas of TE have already taken advantage of such structures to understand the phenomena occurring at the interface of biomaterial-biological entities, applying the concepts into a TE strategy.

Several materials have been developed with osteoinductive and osteoconductive surface topographies intended to lead to the formation of new bone in the case of fracture or disease. Further investigations have shown increased osteoblast functions, such as cell proliferation and activity, when cultured onto nanofeatured surfaces [65]. Moreover, reports have shown that the osteoclasts (bone-resorbing cells) activity [38, 134] was also influenced by the topographical micro- and nanosized cues. The coordination among osteoblasts and osteoclasts is of utmost importance for the effective maintenance of healthy bone. Therefore, results showing synergetic functions of osteoblasts and osteoclasts to ensure a healthy bone remodeling at the implant interface are of extreme importance. Recent studies have reported the successful application of microtopographies in improving the osteointegration and that microgrooves combined with chemical cues can guide selective rat mesenchymal stem cell-derived osteoblasts adhesion and alignment [53]. Further insights on the influence of patterns in the guidance of osteoblast cells were reported by S. Ber and colleagues, showing that through the appropriate choice of surface topography, both the cell alignment and bone formation by mesenchymal osteoprogenitor cells can be enhanced [12]. Polycaprolactone nanofiber meshes obtained from electrospinning have also shown enhanced cell attachment and proliferation when coated with biomimetic calcium phosphate (BCP) layer. [7]. In a different study, it is shown that the combination of nanofibers produced by electrospinning with microfibers originates scaffolds with enhanced characteristics for application in bone tissue regeneration [127]. The innovative structure of these scaffolds, inspired by ECM, simultaneously promotes cell adhesion through a nanonetwork and provides mechanical stability by means of the microfiber mesh. In another study, it was shown that the architecture of nano/microfiber-combined scaffolds elicited and guided the 3D distribution of endothelial cells without compromising the structural requirements for bone regeneration, demonstrating the potential of such structures to overcome the lack of vascularization that is associated with current bone TE constructs [108]. Figure 1.8 shows this scaffold system embedded in a collagen gel, allowing to visualize human umbilical vein endothelial cells (HUVECs) aligned both on the micro- and nanopolymeric fibers.

The advent of micro- and nanotechnologies associated with TE holds a great promise also for cartilage

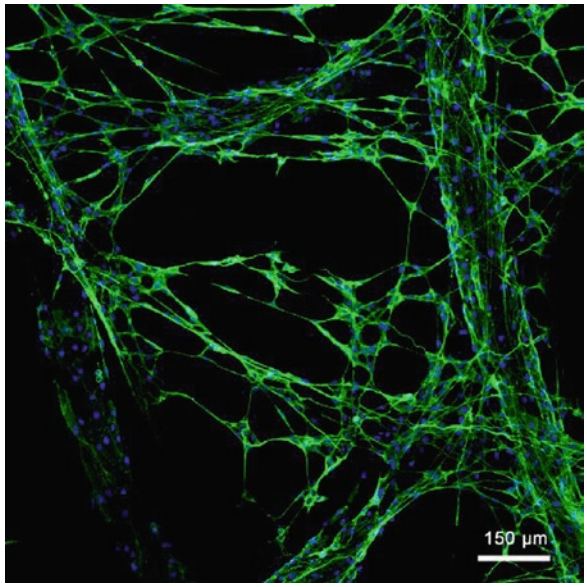



**Fig. 1.8** Human umbilical vein endothelial cells (HUVECs) on collagen-nano and SPCL-microfiber-combined scaffold after 7 days of culture. HUVECs were stained with endothelial-specific marker platelet/endothelial cell adhesion molecule-1 (PECAM-1) and nuclei were counterstained with DAPI

tissue regeneration. Currently, researchers suggest that TE strategies combined with nanopatterned materials can be useful for obtaining functional regeneration of cartilage tissue. The rationale is based on mimicking as closely as possible the natural composition and properties of cartilage. A study reports that micropatterned hyaluronic acid (HA) surfaces induced higher adhesion, migration and alignment of knee articular cartilage chondrocytes when compared to homogenous surfaces. Moreover, the patterned surfaces were shown to promote cell differentiation into chondrocytes [11]. Similarly, Erik Petersen and his colleagues reported that cells cultured onto a microarray of micropatterned surfaces maintained their morphology and their ability to retain important phenotypic aspects of the chondrocytes [94]. Besides being applied into static cultures, micro- and nanotechnologies have also been applied for the understanding cell response to the dynamic changes of the extracellular osmolality. P. Grace Chao and colleagues have described a system that yielded new information regarding the dynamic osmotic loading of chondrocytes, which could also contribute with new insights about the mechanisms of cellular homeostasis of other cell types [24]. Also, electrospinning has been successfully applied to the development of nanofiber meshes of starch-compounded PCL to act as a scaffold for cartilage TE [4].

The behavior of vascular cells (such as endothelial and smooth muscle cells) has shown to be enhanced on micro- and nanostructured surfaces. Microfluidic networks have been developed to produce highly uniform flows that mimic physiological patterns [9]. Sachin C. Daxini and colleagues have devoted efforts into testing the hypothesis that by creating well-defined microtextured surfaces, low shear-stress regions could be created, which would help in retaining endothelial cells. They have shown that endothelial cell retention was significantly improved on micropatterned surfaces when compared to unpatterned ones, which would be beneficial for reducing the thrombogenicity of implanted vascular grafts [34]. Another study went deeper into the analysis of how endothelial cells respond to the shear stress caused by the blood flow. This study helped in understanding the process of endothelial mechanotransduction, suggesting that the design of new substitutes for vascular TE should not only consider the material and biological cues, but also the hemodynamic profiles, in order to improve tissue integration and regeneration [74]. An essential parameter in vascular TE is the recruitment of cells from the blood to the vascular wall in a situation of thrombosis or inflammation. In this context, Divya D. Nalayanda and colleagues have reported the development of microfluidic patterned surfaces that were successful in controlling the adhesion and rolling of endothelial cells under physiological flow [91].

The ability to direct cell attachment and orientation, with the possibility to create fluidically isolated compartments, points out to the distinctive advantage of micro- and nanotechnologies for neural tissue regeneration over the regular culture conditions. A very promising work that went deeper into the issue of synaptic signaling presented results that established that synapses forming on confining geometry of the micropattern are physiologically normal and capable of performing plastic modulations, demonstrating their usefulness as a model for signal processing by neuronal networks [130]. Essential parameters within a neural TE strategy are being further exploited by means of micro- and nanotechnologies, such as the cell orientation, allowing for mimicking the microcircuitry that is encountered in the native tissue. A work of Christopher Bettinger and coworkers reports the use of a flexible and biodegradable substrate of poly(glycerol–sebacate) with rounded features that could further elucidate the mechanism of cell alignment and contact guidance [14].

The liver is another well-studied system in TE since the existing systems cannot fully mimic the synthetic and metabolic liver system. One option is the development of liver-like structures by means of micro- and nanotechnologies. Recently, a very promising device was developed by scientists at the Harvard Medical School and the Massachusetts Institute of Technology that could eventually be used to create a functional liver organ. Basically, it involves the development of a network of microscopic tubes that branch out in a pattern similar to a vascularized system, to provide oxygen and nutrients to the liver cells. Indeed, 95% of the liver cells survived up to 2 weeks in this system [19]. Another system that combines microtechnologies and TE is the one described by Bhatia that consists of a miniaturized, multiwell culture system with a micropatterned architecture of collagen that has shown to maintain the phenotypic behavior of primary rat hepatocytes [59]. Recently, a microfluidics bilayer device with a physiologically based network was also described. The device showed to be able to maintain

hepatic functions of both human hepatoma cells and primary rat hepatocytes [22].

The application of micro- and nanotechnologies in the biological field has recently gone beyond the mainstream research for the engineering of a specific tissue. Nowadays, stem cells and biomaterials that support their adhesion, proliferation, and differentiation represent a very intense area of research. The use of micro- and nanotechnologies for stem cell adhesion, proliferation, and/or differentiation is a slightly different approach in comparison to the aforementioned tissue engineered approaches. By using stem cells, researchers aim the regeneration of not only one specific tissue but also a wide variety of tissues and organs. In fact, micro- and nanotechnologies are of extreme importance for studying stem cell differentiation since they allow generating a tightly tailored microenvironment according to the specificities of each tissue. Recently, Khademhosseini and collaborators have successfully developed cell-repellant poly(ethylene glycol) (PEG) wells that were used as templates for the formation of embryoid bodies (EB), which are cell aggregates of embryonic stem (ES) cells. This promising method showed that microwell techniques can be of great use for initiating the differentiation of EB under controlled microenvironments [50]. Another study showed the successful application of micro- and nanotechnologies for the maintenance of the undifferentiated state of human embryonic stem (hES) cells. The microwell-patterned surfaces have proven to be effective in generating almost perfect aggregates for further differentiation studies [56]. Bhatia and coworkers have been devoting a great effort into the development of high-throughput platforms for the analysis of the signals and mechanisms that regulate stem cell fate [15]. Recently, a platform for assessing the interaction of ECM bioentities and growth factors and their effect on stem cell fate was developed [39]. This high-throughput technology allows for the simultaneous analysis of 1,200 different experiments. The differentiation along cardiac lineage was assessed by means of a confocal microarray scanner. This technology represents a step further on the understanding of the microenvironment that dictates stem cell fate [39].

Besides the studies with ES cells, reports combining stem cells from other sources and micro- and nanotechnologies have shown really promising results. An example is the work described by D. Zahor and colleagues [149] who have induced topography-guided alignment of mesenchymal stem cells by culturing them on micropatterned silicon surfaces. Another study evaluated the efficacy of a micropatterned surface in differentiating multipotent mouse bone marrow stromal precursors into fat tissue [26]. The results showed the positive influence of the patterns in lipid production. Recently, the development of a biomimetic scaffold that could mimic a specific niche, where bone marrow-derived hematopoietic stem cells (BM-HSCs) could healthily proliferate and differentiate, was also reported [75].

### 1.4.2 Towards 3D Micro and Nanofabricated Structures

An effort has been made to push the domain of micro- and nanotechnologies from bi-dimensionality to three-dimensionality [82] extending the applicability of these technologies. This transition to 3D structures can be achieved using micro- and nanotechnologies to buildup three-dimensional structures by stacking multiple two-dimensionally microfabricated layers. This kind of technology has been widely used in TE strategies for developing micro- and nanogrooved three-dimensional microfluidic devices for the replication of several kinds of tissues [131]. Despite the high resolution achievable through those techniques, the basic two-dimensional nature of the substrates used has not yet allowed to fully replicate the real three-dimensional structure of tissues, and consequently, to replicate their full functionality.

Some of the above-mentioned techniques have already been successfully applied in the development of 3D supports for cell growth. For example, a method that combines the chemistry of bottom–up approaches with the engineering of top–down approaches was successfully employed to the development of 3D hydrogel structure [35]. Indeed, of the many synthetic materials being explored, hydrogels are among the most widely adopted for 3D cell cultures, due to their high water content and mechanical properties. Top–down TE approach allows for constructing 2D patterned hydrogels, either by photolithography or soft lithography methods. A bottom–up approach is used further for the modular assembly of the small building blocks. A good example of this combined process is the packing

of rod-shaped collagen microgels seeded with HepG2 hepatocytes and cocultured with endothelial cells at the surface [128]. Langer and collaborators developed structures with defined shapes by micromolding photocrosslinkable hydrogels. Several cell types were encapsulated and the architecture tightly controlled [54, 147]. Using a different approach with photopolymerizable PEG hydrogels, it is possible, with tailored chemistry and architecture, to generate a 3D structure that can further support hepatocyte survival and liver-specific function [126].

Conceptually, 3D microfabricated objects should be created with three-dimensional resolution and ideally be freely manipulated (i.e., fabricated without any necessary attachments to the substrates). One of the challenging issues in TE strategies is still the uniform distribution of cells within 3D-scaffolds. As an attempt to surpass this challenge, the effect of three-dimensional, porous poly(L-lactide-co-glycolide) (PLGA) scaffolds modified with poly(ethylene oxide) (PEO) on cell behavior within a bone TE strategy was studied. This work showed that patterned regions of low and high cell adhesion were demonstrated on scaffolds fabricated with 1 mm thick stripes of PEO and non-PEO regions, respectively [62]. Another very promising work was recently reported [55], which is based on using microfluidic patterning for the development of 3D cardiac organoids. After seeding, the cardiomyocytes elongate along the HA patterns that serve as inductive templates for organoid assembly. This study has further shown that only after 3 days of culture, the cardiomyocytes detach from the surface and start to show a contractile behavior.

Many groups are now investigating the possibility of creating 3D objects through rapid prototyping techniques which consist of building up layer upon layer of material by printing methodologies. This approach is already successfully used in large-scale industrial rapid prototyping by using techniques such as inkjet printing, stereolithography, selective laser sintering, or fused deposition modeling. Such technologies are capable of generating objects with high geometric freedom and possessing highly complex three-dimensional architectures. Prototyping techniques based on the material selective deposition are also capable of building structures from actual living tissues/cells. The technology, so-called bioprinting, offers the ability to deposit cells and other biomolecules in a rapid layer-by-layer method, allowing creating three-dimensional tissue-like structures. A complex three-dimensional microfluidic system was fabricated with PDMS by rapid prototyping using two-level photolithography and replica molding. This method allows generating complex patterned microfluidic systems [5].

The 3D bioprinting technology is currently being studied for possible use in TE applications where organs and body parts are intended to be built using inkjet techniques [85]. The above-mentioned techniques, although capable of being fast and economic, are mostly limited by the scale factor since, in most cases, they are not capable of applying high printing resolutions.

Ultrasmall features may be achieved by the 3D microfabrication technique of 2-photon polymerization. In this approach, the desired 3D object is traced out in situ (in the interior of a photopolymerizable liquid gel) by a focused laser beam. Unlike the commonly used lasers, the laser source used in this methodology is based on a visible light, ultrashort pulse laser. The gel is cured to a solid only in the places where the laser is focused, due to the nonlinear nature of photoexcitation. When the process is finished the remaining gel is simply washed away. Feature sizes with 700 nm can be produced by this method, which also allows the production of complex structures including moving and interlocked parts [52]. Another important feature of this technology is the nature of the polymerizable gel which can be made of a range of proteins such as fibrin, collagen, and albumin which are very abundant in native human tissues.

### 1.4.3 Towards In Vivo Microenvironment: Microbioreactors

Several recent studies demonstrate the importance of mimicking in vitro certain critical aspects present in the native environment of tissues to be regenerated. Therefore, besides the basics of TE strategies, scaffolds, and cells, it is of utmost importance to provide the cell-scaffold constructs with the appropriate microenvironment that will encourage effective organization among the elements of a TE strategy. The most widely used type of culturing system operates in static culture conditions. However, it is known that it may cause nonhomogenous distribution of cells and nutrients and does not allow mimicking the flow stresses that are present in vivo. Bioreactors are systems that have shown to be successful in surpassing these challenges in culture

systems. Bioreactors used in TE strategies not only allow the growing of cells to higher densities but can also be used as extracorporeal devices for liver and kidney diseases. Moreover, in these systems, the biological and biochemical processes are closely monitored and the culturing conditions, such as the pH, temperature, pressure, shear stress, nutrients supply, and waste removal, are tightly controlled.

Microbioreactors, also known as microfluidic bioreactors, offer further advantages for cellular applications. Besides providing large surface-area-to-volume ratio, they can offer microscale controllable fluid circuits [72, 139]. Indeed, microreactors have shown promising results in applications where conventional bioreactors have failed, since they act not as a mere culturing system, but as a device for studying the mechanisms occurring at the tissue microenvironment. This allows designing better materials and/or culturing systems. For instance, a perfusion-based, micro 3D cell culture platform was designed and fabricated, based on SU-8 lithography and PDMS (polydimethylsiloxane) replication processes and used to study the loading of cell/agarose constructs. Moreover, this system was found to be particularly useful for cell culture-based drug screening systems [138]. In fact, cell culture assays, combined with microbioreactors are now emerging as enabling tools for high-throughput cytotoxicity assays, since they allow for high controllability of operation and on-line monitoring/sensing [146]. A different system demonstrated the long-term culture (more than 2 weeks) of mammalian (human foreskin fibroblasts, HFF) cells in a microbioreactor under constant perfusion and the importance of understanding the relationship between design parameters (channel size, oxygen lever supply, shear stress, flow rate) and cell behavior (cell growth, cell morphology, perfusion rate) in microscale culture system [63]. In another approach, a microbioreactor with the size of a glass slide was used for studying the mechanisms involved in culturing in vitro human embryonic stem cells (hESCs) [30]. A different microbioreactor with a PDMS-treated surface was developed based on mass transport simulation. The elastomeric surface was treated with a surfactant in order to diminish the non-specific protein adhesion, keeping the culture conditions steady. This system helped in understanding the kind of environment that provides better chondrocytes stable culture conditions over the culturing period [140]. Within a liver TE strategy, two types of PDMS microbioreactors containing a membrane, used as a scaffold for the attachment of cells, were developed. In this system, the cells were immersed in the culture medium, expressing much higher functions and better mimicking the hepatocytes in vivo. After 15 days of culture, the primary adult rat hepatocyte cultures have shown good cell attachment and reorganization, revealing to be promising tools for future liver TE [92]. Also, aiming at promoting liver tissue regeneration, Linda Griffith and coworkers have developed a microbioreactor for perfused 3D liver culture. The reactor dimensions were designed in such a way that the flow rates meet the estimated values of oxygen demand and also provide a shear stress at or below the physiological one. For 2 weeks, primary rat hepatocytes rearranged to form tissue-like structures, indicating that this system approximates the perfusion and architectural properties of an in vivo hepatic tissue [98, 99]. Recently, an array of twelve microbioreactors was reported [21]. With this technology, high-throughput assays can be performed, by assessing many factors that regulate cell behavior. The microbioreactor was fabricated by soft lithography and each individual microbioreactor is perfused by a culture medium. Moreover, this system allows for cells to be cultured onto the substrate or encapsulated in hydrogels, being the results followed by automated image analysis. As a proof-of-concept, researchers have cultured C2C12 cell line, primary rat cardiac myocytes, and hESCs, illustrating the utility of the microbioreactors array for controlled studies.

A variety of large-scale bioreactors have been developed and optimized for TE strategies. Nevertheless, microfluidics has lead to the advent of a new generation of bioreactors aimed at mimicking in vitro the native microenvironment of tissues. Despite the already performed research in this area, microbioreactors are a relatively new direction in TE, still leaving a plenty of room for new developments in human 3D models for studying the underlying mechanisms in cellular microenvironments.

## 1.5 Expert Opinion

Micro and nanotechnologies have been emerging as useful tools both for developing structures for TE strategies and for studying in vitro the in vivo microenvironment