Chapter 14

Alignment of Cells and Extracellular Matrix Within Tissue-Engineered Substitutes

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Additional information is available at the end of the chapter

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1. Introduction

Most of the cells in our body are in direct contact with extracellular matrix (ECM) components which constitute a complex network of nano-scale proteins and glycosaminoglycans. Those cells constantly remodel the ECM by different processes. They build it by secreting different proteins such as collagen, proteoglycans, laminins or degrade it by producing factors such as matrix metalloproteinase (MMP). Cells interact with the ECM via specific receptors, the integrins [1]. They also organize this matrix, guided by different stimuli, to generate patterns, essential for tissue and organ functions. Reciprocally, cells are guided by the ECM, they modify their morphology and phenotype depending on the protein types and organization via bidirectional integrin signaling [2-4]. In the growing field of tissue engineering [5], control of these aspects are of the utmost importance to create constructs that closely mimic native tissues. To do so, we must take into account the composition of the scaffold (synthetic, natural, biodegradable or not), its organization and the dimension of the structure.

The particular alignment patterns of ECM and cells observed in tissues and organs such as the corneal stroma, vascular smooth muscle cells (SMCs), tendons, bones and skeletal muscles are crucial for organ function. SMCs express contraction proteins such as alpha-smooth-muscle (SM)-actin, desmin and myosin [6] that are essential for cell contraction [6]. To result in vessel contraction, the cells and ECM need to be organized in such a way that most cells are elongated in the same axis. For tubular vascular constructs, it is suitable that SMCs align in the circumferential direction, as they do in vivo [7, 8]. Another striking example of alignment is skeletal muscle cells that form long polynuclear cells, all elongated in the same axis. Each cell generates a weak and short contraction pulse but collectively, it results in a strong, long and sustained contraction of the muscle and, in term, a displacement of the member. In

the corneal stroma, the particular arrangement of the corneal fibroblasts (keratocytes) and ECM is essential to keep the transparency of this tissue [9-13]. Tendons also present a peculiar matrix alignment relative to the muscle axis. It gives a substantial resistance and exceptional mechanical properties to the tissue in that axis [14, 15]. Intervertebral discs [16], cartilage [17], dental enamel [18], and basement membrane of epithelium are other examples of tissues/organs that present peculiar cell and matrix organization. By reproducing and controlling those alignment patterns within tissue-engineered substitutes, a more physiological representation of human tissues could be achieved.

Taking into account the importance of cell microenvironment on the functionality of tissue-engineered organ substitutes, one can assume the importance of being able to customise the 3D structure of the biomaterial or scaffold supporting cell growth. To do so, some methods have been developed and most of them rely on topographic or contact guidance. This is the phenomenon by which cells elongate and migrate in the same axis as the ECM. Topographic guidance was so termed by Curtis and Clark [19] to include cell shape, orientation and movement in the concept of contact guidance described by Harrison [20] and implemented by Weiss [21, 22]. Therefore, if one can achieve ECM alignment, cells will follow the same pattern. Inversely, if cells are aligned on a patterned culture plate, the end result would be aligned ECM deposition [23].

The specific property of tissues or materials that present a variation in their mechanical and structural properties in different axis is called anisotropy. This property can be evaluated either by birefringence measurements [24, 25], mechanical testing in different axis [26], immunological staining of collagen or actin filaments [23] or direct visualisation of collagen fibrils using their self-fluorescence around 488 nm [27, 28].

Several techniques have been recently developed to mimic the specific alignment of cells within tissues to produce more physiologically relevant constructs. In this chapter, we will describe five different techniques, collagen gel compaction, electromagnetic field, electrospinning of nanofibers, mechanical stimulation and microstructured culture plates.

2. Methods to align cells and ECM in tissue-engineered constructs

2.1. Collagen gel

Collagen is the main constituent of the ECM [1], it is therefore logical to use it as a scaffold for tissue engineering [29, 30]. Collagen is produced by cells as a protocollagen strand that, upon enzymatic modification, will be able to assemble into procollagen triple helix and then into tropocollagen triple helix. Self-assembly of tropocollagen into collagen microfibrils is followed by lysyl oxidase crosslinking to finally form a collagen fiber [31]. For tissue engineering applications, collagen can be extracted from dermis of different species including bovine, porcine, avian and human [32, 33] and tissues such as human placenta [34]. Collagen monomer can be bought commercially from different providers. However, these solutions do not always contain the full-length collagen molecule, which can affect its properties.

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Preparation of a collagen gel from these solutions is quite simple, the collagen solution, provided at pH 2, self-assemble at 37°C and at a neutral pH. Cells can be added to the solution after neutralisation and before casting of the gel, allowing for a uniform distribution of cells in the construct. As cells migrate and elongate into the gel, they will try to anchor themselves to the collagen fibers, but since the collagen gels are soft, cells will deform the fibril network causing the gel to compact [35]. If this contraction is controlled by applying a static constraint in one direction, the gel will contract differentially in the constrained and unconstrained axis. This will result in an anisotropic construct because collagen fibers and cells will become aligned in the constrained axis [34, 36, 37].

Thomopoulos et al. [28] used fibroblast populated collagen gels that were constrained either uniaxially or biaxially to evaluate the anisotropy generated in the gel. Uniaxial static strain resulted in gel contraction in the unconstrained axis and lead to a structural and mechanical anisotropy. They found no difference between tendon fibroblasts and cardiac fibroblasts in anisotropy generated in the construct. They also demonstrated that active remodeling of the gel by cells is not necessary for the development of anisotropy in collagen gel. Indeed, uniaxially constrained collagen gel without cells also become anisotropic. This surprising result could be explained by the force generated by collagen polymerisation [38]. They also developed a mathematical model to predict anisotropy in fibroblast-populated collagen gels [39]. They found that mechanical anisotropy could not be explained solely by collagen fibers alignment, but also take into account the redistribution of collagen fibers upon remodeling, nonaffine fiber kinematics [40, 41] and fiber lengths. Costa et al. [42] also investigated the mechanism of cell and matrix alignment in constrained collagen gels. They constrained fibroblast-containing collagen gels with different shapes (square, triangle and circle) and liberated one or more of the edges to create anisotropy into the gel. Contrasting with a previous report of Klebe et al. [43], they showed that fully constrained gels present random cell and matrix orientation. Nevertheless, on the basis of the results obtained in their study, Costa et al. proposed that rather than aligning along the local direction of greatest tension, cells orient parallel to the local free boundary. An interesting result was obtained with the round shape constrain. As expected, no alignment were present when gel was uniformly constrained, but when they cut a central hole in the construct, the gel contracted away from the central hole and cells aligned in the circumferential orientation. This result is consistent with previous results obtained in blood vessel reconstruction in which a collagen gel is contracting around a central mandrel causing circumferential cell alignment [34]. Grinnell and Lamke [44] cultured fibroblasts on hydrated collagen lattices. They found that cells reorganized the network and aligned the collagen fibrils in the plane of cell spreading, becoming more densely packed. They noted that the lattice has thinned to one-tenth of its original thickness.

Weinberg and bell [45] used collagen gel seeded with bovine cells to produce the first tissue-engineered blood vessel. This weak construct was supported by a Dacron mesh in order to sustain physiological pressure. This method was improved later by other groups to enhance mechanical properties of the collagen gel to get rid of the synthetic material, but those constructs were still too weak to be implanted. L'heureux et al. [34] produced such a tissue-engineered blood vessel using collagen gel. The gel was made of a mixture of human type I

and III collagen at a final concentration of 3 mg/ml, it was seeded with human SMCs and finally casted around a cylindrical mandrel, forming the media layer. The anisotropic strain generated by the mandrel constraining gel compaction, combined to a manual detachment of the gel adhesion to the mandrel, caused a progressive circumferential alignment of the SMC. The same process was repeated to produce the adventitia, using human dermal fibroblast embedded in collagen gel and casted around the media layer. Contrasting with the result obtained with the SMC media layer, fibroblasts of the adventitial layer did not get self-oriented. This type of construct was still too weak to be implanted. This paper also showed that gel compaction speed is influenced by initial cell seeding concentration in the construct.

Collagen gel has also been used to engineered intervertebral disc. The highly organized annulus fibrosus which present a cell and matrix alignment, is contrasting with the nucleus pulposus showing random organization. Bowles et al. [16] isolated cells from both parts of the intervertebral disc and seeded them into collagen gels. Interestingly, cells kept their capacities to become organized or not when cultured in vitro and seeded into collagen gel constructs. Robert Tranquillo published several papers using collagen gel, mostly on engineered vascular constructs. Some of his work was done using magnetic alignment of fibers, it will be discussed in the following section. In 1992, they [46] described mathematical theories to understand the complex coupling of cell and matrix deformation in collagen gel populated with cells.

Thus, alignment of cells in collagen gel was one of the first cell alignment method applied to tissue engineering. It is easy to perform, relatively inexpensive and gives interesting results for specific applications. On the other hand, collagen gel do not show mechanical properties sufficient for load bearing application such as bone, cartilage, ligament and blood vessels, at least if it is used alone.

2.2. Electromagnetic field

Electromagnetic field (EMF) can affect multiple aspects of cell behavior and ECM remodeling. We are always in contact with EMF, either coming from the earth, high voltage lines or mobile phones [47, 48]. It is still unclear whether EMF are linked or not to health problems such as cancer, but this hypothesis seems unlikely in most cases [49]. Even if mobile phone usage is probably not linked with brain cancer, the capability of EMF to influence cells and extracellular matrix are clearly demonstrated [50-55]. It is therefore crucial to investigate this relation to first prevent potential deleterious exposure that might lead to health problem and second, to understand mechanisms and eventually develop novel medical therapies [56-58] as well as tissue engineering applications [59-63]. When exposed to a strong EMF, collagen, as well as other biomolecules such as fibrin, will align perpendicularly to the field. This phenomenon is caused by the negative diamagnetic anisotropy of the collagen molecules [64]. This property causes the polymerisation process to take place in a particular orientation. Tissue engineers have used this property to produce alignment of ECM scaffolds and cells in different reconstructed tissues. EMF was also shown to induce orientation of cells, including epithelial cells [65-67], fibroblasts [65, 68], erythrocytes [69] and osteoblasts [61, 70].

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The tendency of biomolecules to align in an EMF has been demonstrated more than 30 years ago, but tissue engineering applications have arisen more recently. Strong EMF were proposed as a method to align fibrin polymer by Torbet et al. [71]. They reported that polymerisation of fibrin gels under a strong EMF resulted in oriented fibrin polymerisation. They also speculate right when proposing that this technique could be extended to other polymers and to living cells as it was done afterward. Twenty-six years later, the same researcher [72, 73] used EMF to align collagen fibers, in order to replicate the physiologic structure of the corneal stroma. This structure possesses a particular arrangement of aligned collagen fibers that cross each other orthogonally. This pattern was recreated by cycle of gelation-rotation-gelation of type I collagen under a 7 Tesla (T) EMF. When corneal fibroblasts, or keratocytes, are seeded in the construct, they align themselves by contact guidance in the local orientation of the scaffold. This technique was used to produce a hemi-cornea, composed of the stroma and the epithelial layer and showed promising results when grafted on an animal model [62]. Even if this represents a significant advance in corneal tissue engineering, the resulting corneal stroma substitute presents a slightly different geometry than native cornea. In a non-pathological cornea, the arrangement of orthogonal lamellas within the stroma consist of a mesh of orthogonal collagen lamellas that are woven together in multilayer. This arrangement provides the cornea with a strong mechanical resistance while remaining a transparent structure [74-77]. Reproducing this geometry over the normal thickness of the stroma seems quite difficult using this layer-by-layer technique. Kotani et al [70] studied the effect of EMF on bone formation and orientation. They have shown that, when exposed to a strong EMF of 8 T, osteoblasts oriented parallel to the field. In contrast, when osteoblasts and collagen are mix together, the alignment of both constituents is perpendicular to the field, as for collagen alone. This supposed that contact guidance is a stronger inducer of cell alignment than EMF. Two years later, [61] they showed that exposure of mouse osteoblasts to a strong EMF improved differentiation and matrix synthesis in vitro. They also demonstrated that ectopic bone formation in vivo is stimulated by EMF. When pellets of collagen (1.2 mg/pellet, bovine) containing bone morphogenic protein 2 (5 μg/pellet, human recombinant) are implanted subcutaneously and exposed to an 8 T EMF, bone formation was exacerbated and aligned parallel to the EMF.

Robert Tranquillo used EMF as a method to align cells and ECM in tissue-engineered constructs [59, 63, 78, 79], with a particular focus on media substitutes. In 1993, Guido et al. [24] developed a quantitative method to study cells and fibrils orientation when submitted to an EMF. This method used time-lapse image analysis and live automated birefringence measurements to quantify this phenomenon. Tranquillo et al. [59] showed that a 4.7 T EMF could orient collagen fibrillogenesis resulting in a circonferential orientation, in order to create a media equivalent. Barocas et al. [80] compared four different fabrication conditions for a media equivalent composed of SMC embedded in a collagen gel that were either submitted to EMF and/or mandrel compaction. Compaction of the gel around a central mandrel by SMCs induced a circumferential alignment of cells and collagen fibers, as demonstrated previously [34]. Magnetic circumferential alignment was performed prior gel compaction to produce prealigned gels. When those gels were allowed to contract freely, the circumferential alignment was lost, but when a mandrel was present, the alignment was better than with EMF

alone. This method can also be used to guide neurite outgrowth of neural cells. Dubey et al. used collagen [81] and fibrin [82] aligned gel to control outgrowth of neurite. When fibers were aligned, neurite outgrowths were stimulated and could therefore grow longer than random aligned controls.

Retrospectively, this method is effective for biological scaffold such as collagen and fibrin, but to our knowledge, alignment of synthetic materials such as poly(glycolic acid) (PGA) or poly(D,L-lactide-co-glycolide) (PLGA) has not been performed yet. This technique requires a special apparatus capable of generating a strong EMF. Cell viability does not seem to be affected by EMF, allowing for a uniform cell distribution in the construct.

2.3. Electrospun nanofiber

Electrospinning of nanofibers is an interesting approach to produce scaffold for tissue engineering [83-89]. This technique can be used to produce aligned scaffold that will dictate cell elongation by contact guidance [90]. The process of producing polymer microfiber using electrostatic forces was patented in 1934 by Formhals [91] but tissue engineering applications such as musculoskeletal [92] and vascular [93] has been developed recently. Electrospinning can be performed with simple setup consisting of a syringe pump, a high voltage source, and a rotating collector [85]. Precise description of the different possible setups and techniques have been reviewed in details previously [94]. Briefly, a polymer solution is hanging at the tip of a syringe needle by surface tension. When an electric current is applied, EMF results in charge repulsion within the polymer solution, causing the initiation of a jet. Solvent evaporate while jet is traveling, resulting in polymerisation into fibers, which are captured by a collector [94]. Depending on settings and polymers used, those fibers can range from 3 nm to greater than 5 μm in diameter [95]. This technique has been used to engineer many types of scaffolds for tissue engineering [90] including synthetic polymer such as poly(D,L-lactide-co-glycolide) (PLGA) [96], poly-(ε-caprolactone) (PCL) [97], 50:50 poly(L-lactic acid-co-ε-caprolactone) (PLCL) fibers [98], or natural polymer such as collagen [99] or fibrin [100] for various tissue applications. It is also possible to create composite scaffolds by spinning different polymer solution either together or consecutively on the same target. Due to the great plasticity of the technique, it is simple to engineer different patterns to guide cell fate in the desired direction. In order to do so, a rotating mandrel can be used to collect the fiber, resulting in aligned nanofibers [101].

Jose et al. [102] developed an aligned nanofibrous scaffold for bone tissue engineering. This scaffold is a nanocomposite copolymer of PLGA and nano-hydroxyapatite (HA). Fiber diameters, glass transition temperature, storage modulus and degradation rate were characterized for different concentration of nano-HA from 0 to 20%. Briefly, average fiber diameters were augmented from 300 nm for PLGA to 700 nm for 20% nano-HA but formed aggregate at those high concentration. Fiber alignment capability was not influenced by nano-HA concentration. Mechanical properties of the composite material were modulated by nano-HA concentration, it acted as a reinforcement agent at lower concentration (1% and 5 %) but induced defects in the structure at higher concentrations (10 % and 20 %). Influences were also seen in degradation rate and storage modulus. In order to show cell compati-

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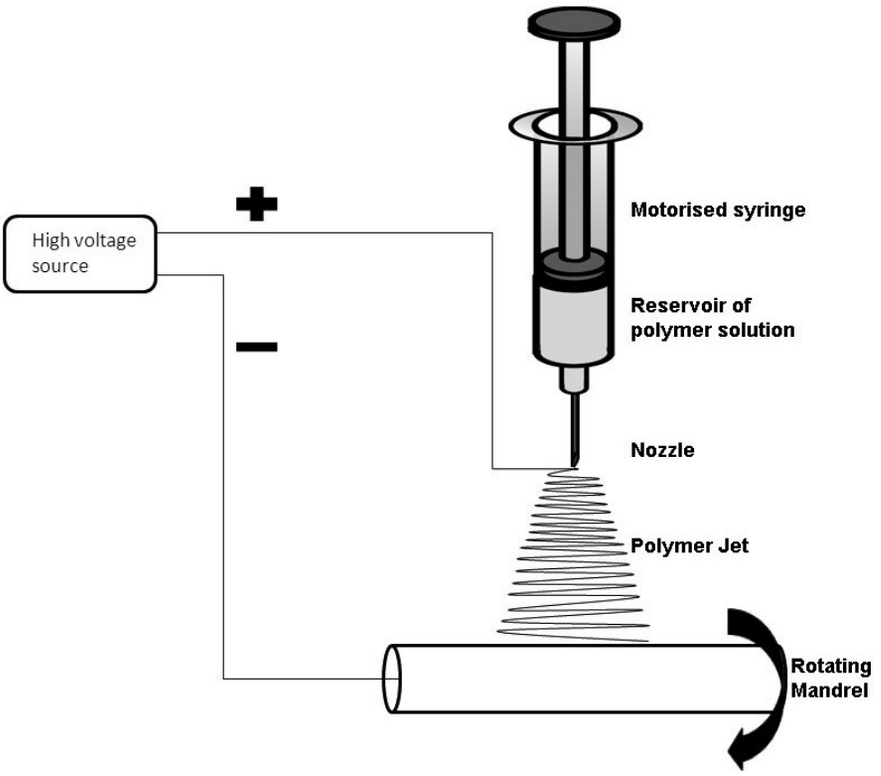

Figure 1. Schematisation of an electrospinning setup. The polymer solution is positively charged while the rotating mandrel is negative. The solution is pushed through the nozzle at constant speed. The solvent evaporates while the jet is travelling, resulting in fibers formation.

bility, a collagen component was added to the PLGA (20:80) [103], and human mesenchymal stem cells (MSCs) were used for the demonstration. Thomas et al. [104] investigated the effect of rotation speed on scaffold alignment, mechanical properties and morphologies. They used a nanofibrous mesh of PCL collected at zero, 3,000 and 6,000 rpm for bone tissue engineering. When the collector rotation speed is increased, more aligned fibers were produced. This resulted in a modification of the morphology and mechanical properties of individual fibers and of the resulting scaffold. Interestingly, the hardness and Young's modulus of individual fibers were diminished while increasing rotation speed, but opposite results were obtained when the whole resulting scaffold was analysed. Ultimate tensile strength of the scaffold in the axis of alignment rises from 2.2 to 9.6 MPa when rotation speed was increased from 0 to 6,000 rpm. This result could be explained by increasing fiber alignment and packing as well as a decrease in inter-fiber pore size when rotation speed is increased. Li et al. [92] performed a similar study on rotation speed. They used electrospun aligned nanofibrous scaffold to control anisotropy into tissue-engineered musculoskeletal constructs. Their scaffolds were also made of a biodegradable PCL polymer, casted on a rotating target to align fibers. Increasing the rotation speed of the shaft from 0 to 9.3 m/s (0 to 7,000 rpm) produced more aligned fibers, to a maximum of 94 % of fibers aligned within a 20° angle range, at maximum speed. Alignment of the ECM leads to an increase in the iso-

tropic tensile modulus ranging from 2.1 MPa for unaligned controls to 11.6 MPa for the aligned substrate at 9.3 m/s. Human MSCs and meniscus fibrochondrocytes, seeded on these aligned scaffolds, attached and elongated in the fibers direction. It is also possible with this technique to produce successive layers at different angles by changing the rotation vector.

Teh et al. [105] produced a silk fibroin (SF) hybrid scaffold for ligament regeneration. Silk is an interesting material for tissue engineering, but the hyper-allergenic sericin component must be removed. It has an interesting degradation rate and show remarkable mechanical properties. Silk was previously used to culture fibroblasts and keratinocytes [106]. The new design proposed by Teh et al. is composed of a bilayered SF, contained a knitted SF fibrous mesh layer and an aligned SF electrospun fibers cast on rotating rods. This construct was seeded with MSCs before rolling it into a cylindrical ligament analog. They found that MSCs differentiation into ligament fibroblast was enhanced by the alignment of the fibers. This resulted in an improvement of tensile properties from 125 to 158 N after 14 days and in an increase of ligament-related protein levels such as collagen I and III as well as Tenascin-c. Xu et al. [93] produced an aligned polymer nanofibrous scaffold for blood vessel reconstruction. They used a biodegradable copolymer of poly(L-lactid-co-ε-caprolactone) [P(LLA-CL)] (75:25), aligned using a rotating disc. They demonstrated that human coronary SMCs elongated and migrated in the direction of the fibers and express a spindle-like contractile phenotype with better adherence and proliferation than on control polymer. Yang et al. [107] used a fibrous scaffold to guide neural cells. They used mouse neural stem cells seeded in a poly(L-Lactic acid) (PLLA) nano/micro fibrous scaffolds made by electrospinning. By using two different concentrations of the PLLA solution, 2% and 5%, they were able to cast nanofibers of 150 to 500 nm and microfibers of 800 to 3000 nm, respectively. They have shown that cell elongation and neurite outgrowth is parallel to fiber's direction. They found that the differentiation of neural precursor cells was higher on nanofibers than on microfibers but independent of cell alignment.

Collagen scaffold produced by electrospinning was done by Matthews et al. [108]. In this paper, they set the basis for electrospinning of collagen fibers for tissue engineering application by varying different parameters (collagen source and concentration, solvent, input voltage). With optimal conditions found in this study, they obtained a matrix containing collagen fibers of 100 nm of diameter that exhibited a 67 nm banding pattern, characteristic of native collagen fibers. They cultured aortic SMCs into this collagen scaffold and obtained uniform distribution of cells into the construct. Zhong et al. [109] went further with collagen nanofibrous scaffolds for fibroblast culture. They used calf skin type I collagen, at 80 mg/ml, electrospunned over a wheel rotating at 15 m/s and formed fibrils of 180 nm, which is smaller than the unaligned ones that have an average diameter of 250 nm. They treated their collagen construct with glutaraldehyde vapor (30%) to enhance the biostability of the scaffold. After seeding the construct with rabbit conjunctiva fibroblasts, they measured cell adhesion, proliferation, morphology and interaction with the scaffold. In addition to cell alignment, they noted a lower cell adhesion but higher cell proliferation on aligned constructs.

Given that electrospinning is a versatile technique to produce aligned ECM for tissue engineering, by modifying the casting parameters (rotation speed, input voltage, distance from

target, dimension of the tip) or the composition of the solution (type of polymer, solvent, concentration) it is possible to produce different structures in terms of fiber diameters and composition. It is also possible to cast successive layers with different orientations to obtain a complex scaffold. This technique will certainly be perfected in the future using computerized and robotized set-up to produce reproducible and complex scaffolds for various tissue engineering applications.

2.4. Microstructured culture plates

As explained above, topographic guidance is the process by which cells respond to the particular arrangement of their environment by modifying their shape and migration vectors [110]. In vivo, cell environment is composed mostly of native collagen molecules and proteoglycans, in vitro, researchers tried to mimic the cues given by proteins to influence cell fate. Those cues can come from different structures resembling or not collagen molecules and have been discuss in previous pages. In the following section, it is the very structure of the culture plate that dictates the organization of cells. To do so, gratings of various dimensions and shapes are created by various methods into a cell-compatible plastic and cells are seeded over it.

Among the different methods to create and control the type and the shape of the guiding structures, there are very interesting and versatile approaches using microfabrication processes analog to the ones developed by the microelectronic industry [111, 112].

Structures with features ranging from nano- to tens of microns scale matching both the ECM proteins or cells dimension can now be created. These techniques can be adapted to different polymers for various applications and use either polymer casting, micromachining or thermoforming. This section will focus on the most common process that uses photolithography and hot embossing as an example. Briefly, a pattern is printed in chromium on a quartz plate to form the mask for photolithography. A photoresist is poured over a silicon plate and exposed to UV light under the mask. The resist is then developed in organic solvent to reveal the pattern and obtain the first wafer (master). This master wafer is then replicated in a polydimethylsiloxan (PDMS) mold that is cured into the silicon wafer. PDMS can be used for cell culture or be replicated on another substrate. In order to replicate it, hot embossing with an intermediate epoxy wafer is used to finally obtain the desired pattern in the chosen polymer.

Numerous types of materials could be used for the final cell culture step. Polystyrene is an interesting material due to its great biocompatibility, being used regularly for cell culture flask [113]. Teixeira et al. [114] have shown that corneal epithelial cells take an elongated shape and aligned themselves along with grooves and ridges as small as 70 nm. They tested various lengths of grooves and ridges to show that cell alignment is similar from 70 nm to 850 nm ridges but is reduced with 1900 nm ridges. Those lengths were chosen to match the approximate dimension of the basement membrane features. In another paper [115], they showed similar results using corneal stromal fibroblasts (keratocytes). They obtain 70% of aligned fibroblasts with pitch (sum of groove and ridge) larger than 800 nm where as few as 35% of epithelial cells were aligned in the features direction. This is a logical result consider-

ing how fibroblastic cells elongate and migrate more than epithelial cells, they are therefore more prone or easy to align.

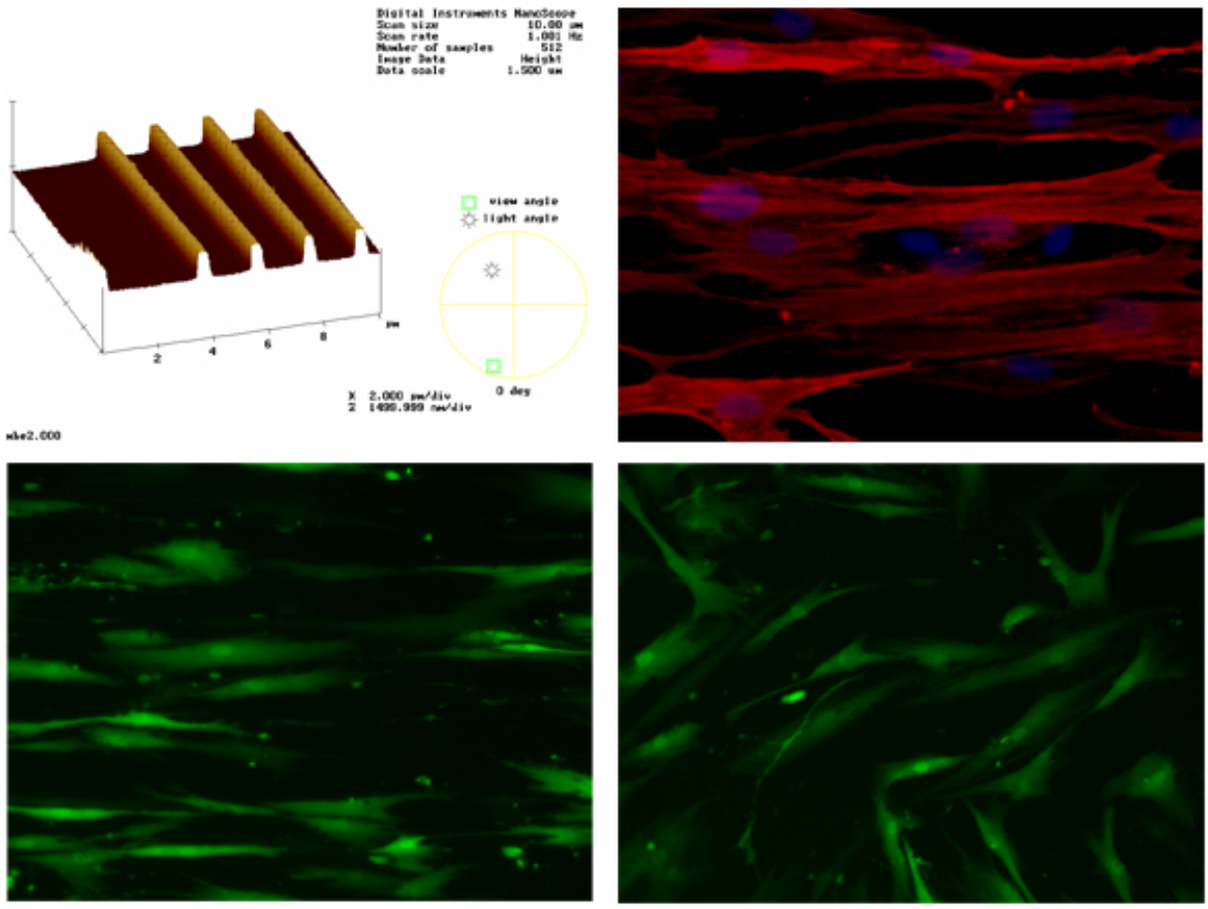

Figure 2. A) Atomic force microscopy of a sample used for replica molding. The master wafer represents the inverse (the negative) of the final results in polystyrene. B) Smooth muscle cells on a microstructured substrate of grooves (1 um) and ridges (4 um) after 1 day of culture. Cells are elongated in the direction of the microstructured pattern. C-D) Human dermal fibroblasts (GFP+) cultured on the same microstructured substrate (C) or on flat plastic (D) after 1 day.

Isenberg et al. [116] cultured SMCs on 50 μm wide and 5 μm deep gratings. They used a combination of photolithography and hot embossing [117] to reproduce patterns of ridges and grooves on polystyrene substrate coated with a thermoresponsive polymer, poly(N-isopropylacrylamide) (PIPAAm) compatible with cell culture [118]. They seeded human aortic SMC on these microtextured culture substrates and allow them to form cell sheets. At room temperature, the cell sheet spontaneously detach from the culture substrate and can be manipulated. In this paper, they have shown that cells elongated and migrated in the direction of the grooves but did not evaluate mechanical or structural anisotropy. In 2012, [119] they tested those parameters and showed mechanical anisotropy in the resulting media layer that mimic the organization of the native vessel.

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Guillemette et al. [23] used photolithography and hot embossing to create a pattern of ridges and grooves (4 μm wide, 500 nm deep) in a thermoplastic elastomer, the styrene-(ethylene/butylene)-styrene block co-polymer (SEBS) [120]. Aligned sheets of SMCs, dermal fibroblasts and corneal fibroblasts were produced using the self-assembly or cell sheet engineering approach [121, 122]. All three cell types aligned in the direction of the long axis of the ridges and grooves, for the cells in direct contact with the substrate. Cells grown with ascorbic acid produced ECM and formed tissues comprising many cell layers [119]. Interestingly, suprabasal cell layer that is in contact with the first cell layer aligned themselves with a shift angle from the previous layer characteristic of the cell type. Guillemette et al. [123] used a combination of micromolding and laser microablation to culture cardiac muscle cells. A pattern of grooves and ridges was designed so that wide grooves could be ablated using a laser to create a porous mesh for cardiac cells to grow. Two different pore shapes were studied, square pores and rectangle pores to increase anisotropy. It was shown that square holes are less effective than rectangle holes in aligning cells within the long axis, and that the combination of rectangle holes and grooves gives better alignment than rectangle holes alone. This demonstration pertains to the classic action of cell alignment on an ECM, and improvement of cell functionality as an end result.

2.5. Mechanical strain

Many cells in the human body are subjected to mechanical stress that dictates their phenotype and orientation. This adaptive process starts within the embryo, but is driven by stress and controlled by morphogen gradients or other cues still to be identified. Mechanical stress is probably an inducer for some cell alignment, such as in vasculogenesis [124].

Cell alignment, induced following compaction of collagen gel around a mandrel [34], is driven by mechanical strain anisotropy. For blood vessel reconstruction, a circumferential constraint causes cell and ECM to align circumferentially, as long as the scaffolding gel could move freely in the longitudinal direction. Nevertheless, there is a particular interest in applying mechanical stimulation to different tissue-engineered constructs including vessel, bone, cartilage and ligament, for the induction of alignment and maturation.

Mechanical strain, as a method to align cells and matrix in order to improve functionality, was extensively used in vascular tissue engineering. Mostly via transluminal pressurisation or via a circulating medium in a specifically designed bioreactor. It has been shown by Kanda et al.[125] that a dynamic deformation of 5% at 60 Hertz of a ring of collagen gel containing SMCs will cause cells and ECM to align in the circumferential direction. Niklason et al. [126] has shown that vascular substitutes, made using a PGA scaffold, submitted to pulsed flow for weeks improved mechanical properties, resulting in a burst pressure of more than 2000 mmHg. In this study, cell and matrix alignment was not investigated. Nerem's team has shown that collagen gels present improved mechanical properties and circumferential alignment of the ECM when submitted to a cyclic deformation of 10% at 1 Hz frequency [127] and this process was driven by the ECM remodeling by matrix metalloproteinase 2 [128-130]

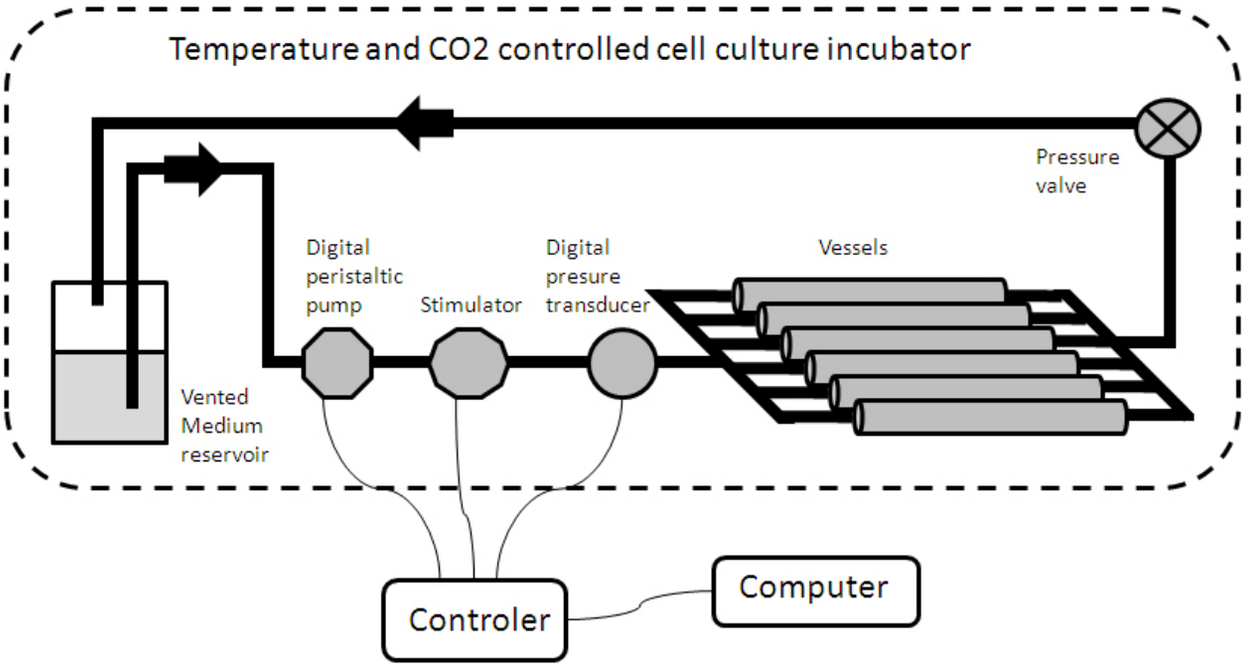

Figure 3. Design of a bioreactor for mechanical strain stimulation of tissue-engineered cylindrical constructs. The culture medium is pumped from the medium reservoir, which is vented through a 0.22 μm sterile air filter, with a computer controlled peristaltic pump. The stimulator is composed of a computer-controlled piston that can generate a cyclic strain in the vessels. The pressure transducer monitors the variation in pressure. The pressure valve ensures an adequate pressure to be built in the vessels. Such devices are commercially available.

Grenier et al. [131, 132] demonstrated that SMCs sheets produced by self-assembly [26, 121, 133] can undergo collagen reorganization following an uniaxial static stretch. More recently, Gauvin et al. [134] extended this experiment using human dermal fibroblast sheets with dynamic stimulation. Constructs were subjected for 3 days to either a static strain of 10%, or a dynamic mechanical stimulation of 10% at 1 Hz. As expected, cells and ECM aligned in the strain axis when submitted to either static or dynamic mechanical stimulation. Ultimate tensile strength and tensile modulus were increased in dynamic mechanical stimulation as compared to unstrained controls and to static strain. Therefore, it demonstrated that mechanical stimulation contributed to establishment of anisotropy in the structure and improves mechanical properties of cell sheet based constructs.

Isenberg et al. [135] evaluated the effects of mechanical strain on media equivalent with an experimental design that isolate its effect from other confounding factors such as gel compaction, creep fatigue and fiber alignment. Therefore, they waited 2 weeks for gel compaction to stabilize, used ribose cross links to fix fiber orientation and to prevent creep deformation. Finally, they placed the construct on distensible latex tubing in a custom designed chamber to produce a pure circumferential strain. They found that modulus and ultimate tensile strength were augmented when the constructs were stimulated for 5 weeks (no change at 2 weeks), at 5% strain, 0,5 Hz and 12.5% duty cycle. Therefore, they demonstrated that independently of the effect on cell and ECM alignment, mechanical strain increased the mechanical properties of collagen based media substitutes.

2.6. Other techniques

There are other techniques that have been developed to align cells and ECM that were not discussed yet. Some of these techniques were too specific or not enough common to give them a complete section, however a quick overview of some of these methods will be given below.

The first method that will be discussed in this section is shear stress. This force is of great importance for blood vessels where it dictates the longitudinal alignment of endothelial cells. This longitudinal configuration is really important for cell function and disturbance in blood flow cause the alignment to be lost. In vessel branching for example, it is associated with endothelial dysfunction, atherosclerosis development and thrombus formation [136]. This capability of endothelial cells to sense the shear stress and align themselves in response to the stimuli rely on specialized cell component called primary cilia [137-139]. This capability can be reproduced in vitro using either a laminar flow chamber [140, 141] or a bioreactor for transluminal flow, either on reconstructed [121] or native [142] endothelium. To induce an alignment response of endothelial cells in vitro, it is important to take into account the parameters that influence the resulting shear stress on endothelium. Those parameters are: vessel section surface, fluid flow and fluid viscosity. Alignment of endothelial cells is influenced by wall shear stress intensity, exposure time and turbidity [110].

A second method, also dependant on shear, was recently developed by McClendon et al. [143] and takes advantage of the biocompatibility of the peptide amphiphile (PA) [144, 145] to produce circumferentially aligned tubes acting as scaffold for arterial tissue engineering. The PA is contained in a liquid crystalline solution that form aligned domains that can be trapped in a gel showing macroscopic alignment by applying low shear rates and ionic crosslink. The interesting point about this technique is the possibility of incorporating cells while forming the scaffold, allowing for full cell penetration into the culture substrate. Therefore, they encapsulated SMCs into their construct and showed that cells proliferated and aligned themselves in the direction of the scaffold, without depending on external stimuli or gel compaction.

Another method for cell alignment is by printing a protein pattern on a culture surface. Cells seeded on these surfaces will adhere and migrate preferentially on the printed pattern resulting in an aligned culture. Thakar et al. [146] used this technique to align vascular SMCs on collagen strip of various width, in order to investigate the relationship between morphology and function of these cells. This interesting technique is difficult to use in a tissue engineering perspective because cells will gradually invade the unprinted region of the surface and therefore alignment of cells will be gradually lost over time.

3. Discussion

The field of tissue engineering has moved forward at great speed in the last two decades, as shown by the rapid augmentation of the number of publication with the terms “tissue engi-

neering" on Pubmed. Researchers have built models for in vitro testing and substitutes that work well within different animal models. On the other hand, there are only a few tissue engineering products that have been tested in humans and even fewer that have moved on as FDA approved product for the market. The words "tissue engineering" allowed retrieval of 46 records on clinicaltrials.gov at the end of august 2012. Beside complications regarding regulatory affairs to get a product accepted, falling in a category between medical device and pharmaceutical drugs [147, 148], some substitutes failed to show adequate mechanical properties to do the job right. Creating more physiological substitutes by recreating the geometry of native ECM and cells is a quite interesting way to improve resistance without introducing a new material or create a thicker construct.

In this chapter, some of the existing techniques that have been published to produce tissue-engineered constructs showing a customised geometry were reviewed. Most of these techniques have been developed for other applications, and adapted later for tissue engineering. Alignment of collagen fibers in collagen gel constrained uniaxially is probably the oldest one, it is a quite simple technique where cell align themselves in the axis of the constraint. This model has been combined with EMF alignment of biomolecules, a more complex technique that direct ECM assembly in a desired orientation. Electrospun nanofibers are becoming more popular in the field and the simple modification of adding a rotating target make it an interesting technique for ECM alignment. The recent advances in microfabrication have made it easy to produce the custom culture substrates that present nanoscale structures at the ECM level. While contact or topographic guidance has been studied quite a while ago, this capability of cells to align themselves in the direction of grooves and ridges of certain dimension is interesting for tissue engineering applications, especially with cell sheet engineering. Finally, mechanical strain is a strong inducer of cell alignment that dictates the geometry of cells in our body. This technique is of great interest for load bearing applications such as cartilage, bone or vascular tissue engineering. Each of the technique mentioned above have advantages and drawbacks, and some of them are dependent on the type of tissue desired.

Constrained collagen gel compaction is a simple technique that is compatible with cell seeding prior gelation, allowing for a uniform cell distribution throughout the construct. On the other hand, collagen gel presents poor mechanical properties, making them unsuitable for load bearing applications such as vascular tissue engineering. The development of hydrogels has helped to partially overcome this problem [29]. Alignment of ECM and cells in EMF is an effective technique but it necessitate state-of-the-art apparatus, not commonly available in a lab, in order to generate an EMF between 4 T and 8 T. As for collagen gels, cells can be added into the solution to have a uniform cell distribution. This technique seems to be restricted at this time to biomolecules, therefore limiting its application field. It can be combined with controlled collagen gel compaction to produce a more potent alignment. Electrospinning of polymer fibers is a very interesting technique that can be applied to a lot of different kinds of polymers by simply modifying the parameters of casting. This technique is particularly effective for tubular constructs since using a cylindrical mandrel as the target will directly create the desired construct. However, it is not possible to seed cells

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while casting the construct. Therefore, cells will need to penetrate the construct on their own, causing cell distribution to be potentially non homogenous if the construct is too thick or not enough porous to allow for cell migration. Microstructured culture plate is an effective technique, especially for cell sheet engineering or self-assembly. This approach relies on the capacity of cells to secrete and organize ECM. When grown on structured substrate, cells will align the extracellular matrix in the direction of the grooves. With this technique, it is possible to create complex design of cell alignment by modifying the surface topography. This is the only method described here that does not rely on a pre-existing scaffolding material. On the other hand, producing those cell culture substrates is long and costly, needing access to a microfabrication clean room containing expensive apparatus. Mechanical strain can be applied by a number of different setups depending on the construct type. This technique is effective to align the whole construct in a preferred direction, but complex patterns cannot be created. The cost of this technique depends on the setting that could be computer-controlled or not. Mechanical stress must be precisely controlled since a strain that would be too strong could lead to creeping of the construct or affect cell viability or ECM integrity.

4. Conclusion

The future of tissue engineering relies on the production of more complex structures, composed of many cell types that will interact together. In order to do so, cells must be assembled together in a structure that mimics their native microenvironment. Techniques to align and organize scaffolds will continue to go forward and new technologies will arise, pushed by the constant need for tissue-engineered constructs for organ transplantation.

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