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Non-thermal Plasma Technology for the Improvement of Scaffolds for Tissue Engineering and Regenerative Medicine - A Review

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Abstract

Non-thermal plasma technology is one of those techniques that suffer relatively little from diffusion limits, slow kinetics, and complex geometries compared to more traditional liquid-based chemical surface modification techniques. Combined with a lack of solvents, preservation of the bulk properties, and fast treatment times; it is a well-liked technique for the treatment of materials for biomedical applications. In this book chapter, a review will be given on what the scientific community determined to be essential to obtain appropriate scaffolds for tissue engineering and how plasma scientists have used non-thermal plasma technology to accomplish this. A distinction will be made depending on the scaffold fabrication technique, as each technique has its own set of specific problems that need to be tackled. Fabrication techniques will include traditional fabrication methods, rapid prototyping, and electrospinning. As for the different plasma techniques, both plasma activation and grafting/polymerization will be included in the review and linked to the in-vitro/in-vivo response to these treatments. The literature review itself is preceded by a more general overview on cell communication, giving useful insights on how surface modification strategies should be developed.

Keywords: Non-thermal plasma technology, surface modification, tissue engineering, scaffold fabrication, biomaterials

1. Introduction

In the 1980's, researchers started first experimenting with the idea of fabricating human tissues. But the true start of the field of tissue engineering was not until 1993, when Langer and Vacanti

wrote their legendary paper. The philosophy behind tissue engineering, as published by its two founders, is the following: "*the loss of failure of an organ or tissue is one of the most frequent, devastating and costly problems in human healthcare. A new field, tissue engineering, applies the principles of biology and engineering to the development of functional substitutes for damaged tissue.*" In the two decades that followed, this new field in science expanded rapidly and continues to do so up to this day. As scientists started to unravel the pathways that drive tissue regeneration, it became clear that the fabrication of fully functioning human tissues was far more complex than initially anticipated [1, 2]. Building a tissue from the bottom up requires a profound knowledge of physics, chemistry, biology, and engineering and this from the macroscale (mm–cm) down to the molecular level (Å). Steady progress has been made over the last 25 years, resulting in some (commercial) successes such as Matrigel® and Dermagraft®. Alongside the rise of the field of tissue engineering, non-thermal plasma technology has gained importance as a cheap and efficient tool for the non-destructive modification of biomaterial surfaces. In this book chapter, an overview will be given on how plasma technology has contributed to the fabrication of better scaffolds for tissue engineering, preceded by an analysis on how to mimic the human body best, down to the molecular level.
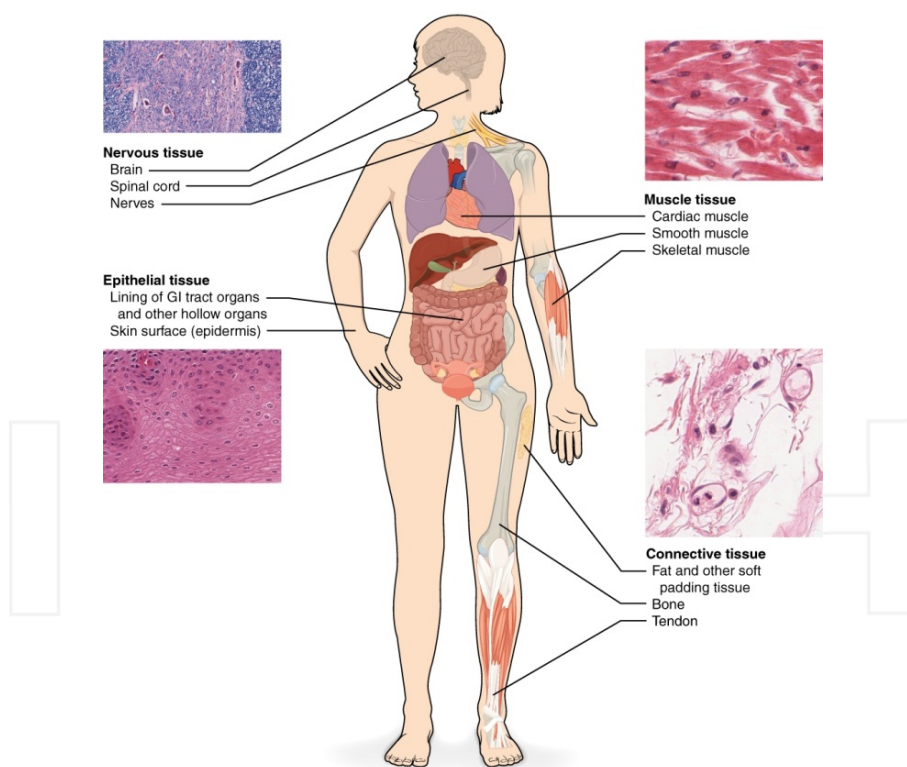

Figure 1. Different types of tissues available in the human body [132].

2. Cellular communication

2.1. Tissue deconstruction

Before understanding that how cells can be manipulated to grow into the required type of tissue, it is important to comprehend what a tissue exactly consists of. In general, a tissue is composed out of (1) a certain set of cells (osteoblast, fibroblast, epithelial cell, neuroblast, astrocyte...) from the same origin, forming the "building blocks" of a tissue, (2) an extracellular matrix (ECM), forming the "cement" of such tissue, offering a 3D structural and biochemical support, and (3) in most cases, a vascular network that allows transport of oxygen and nutrients [3–5]. Depending on the type of tissue (nervous, muscular, epithelial, or connective tissue), both the cell-types, cell ratio, and cell density, as well as the type of matrix (types of proteins and their 3D configuration) will greatly vary, as shown in Figure 1. The key to a viable tissue lies in its communication pathways: How do cells sense their environment? How do cells communicate with each other? What is triggering the different steps in a cell's life cycle? To postulate an answer to these questions, one has to go down to the (sub)cellular level.
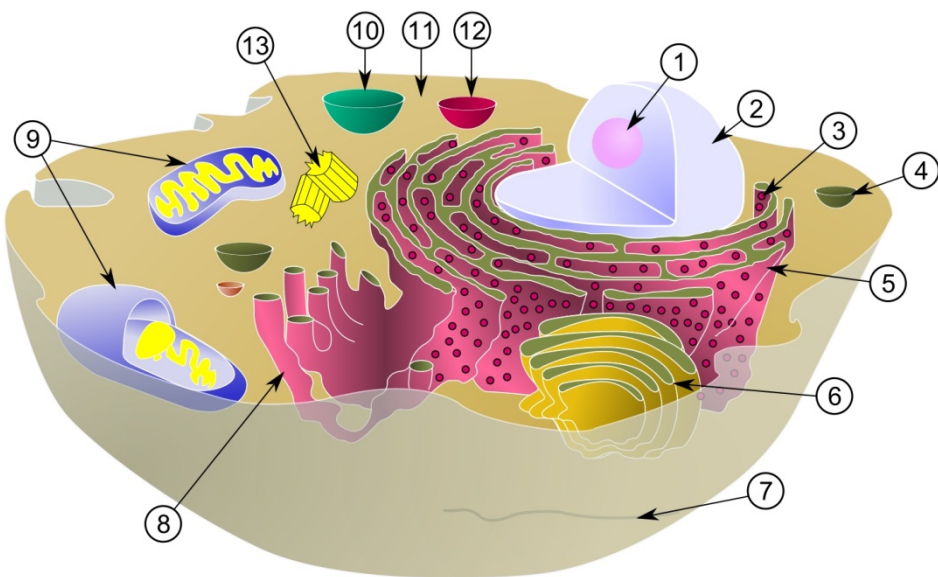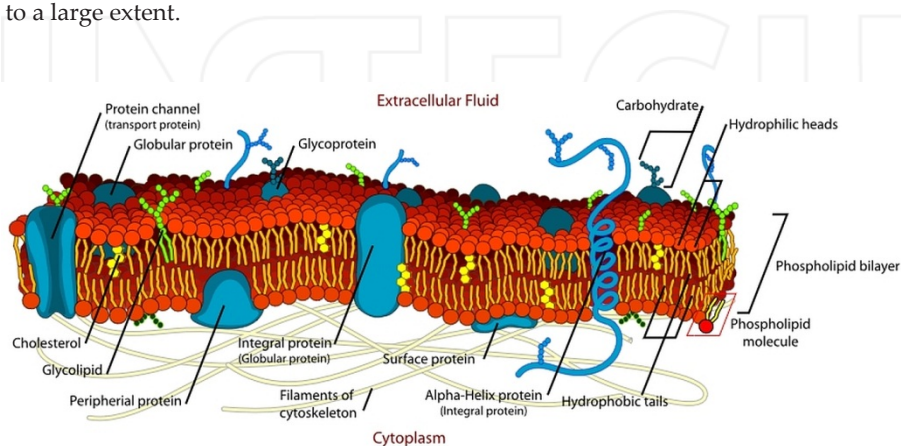

Figure 2. Schematic representation of cellular composition: (1) nucleolus, (2) nucleus, (3) ribosomes, (4) vesicle, (5) endoplasmic reticulum, (6) Golgi apparatus, (7) cytoskeleton, (8) smooth endoplasmic reticulum, (9) mitochondria, (10) peroxisome, (11) cytoplasm, (12) lysosome, (13) centrioles (open source).

2.2. Cell deconstruction

In basic biology, it is taught that a cell consists of a nucleus, containing the genetic information, a number of organelles that are responsible for the cells metabolism, and a cell membrane that

separates and protects the cell interior from its environment (Figure 2) [1, 2, 4–7]. A cell constantly communicates with its environment and with other cells through signals released by these cells. Cell signaling occurs through the cell membrane via transmembrane proteins such as G-protein-coupled receptors and ion exchange channels or via diffusion of lipid soluble molecules and small molecules (Figure 3). Part of the transmembrane proteins consists out of a receptor site at the extracellular side of the membrane. When the matching signal molecule (a protein, a peptide, or an amino acid) binds with such a specific available receptor site, it will trigger a cascade of intracellular physico-chemical pathways that control the behavior of the cell to a large extent.
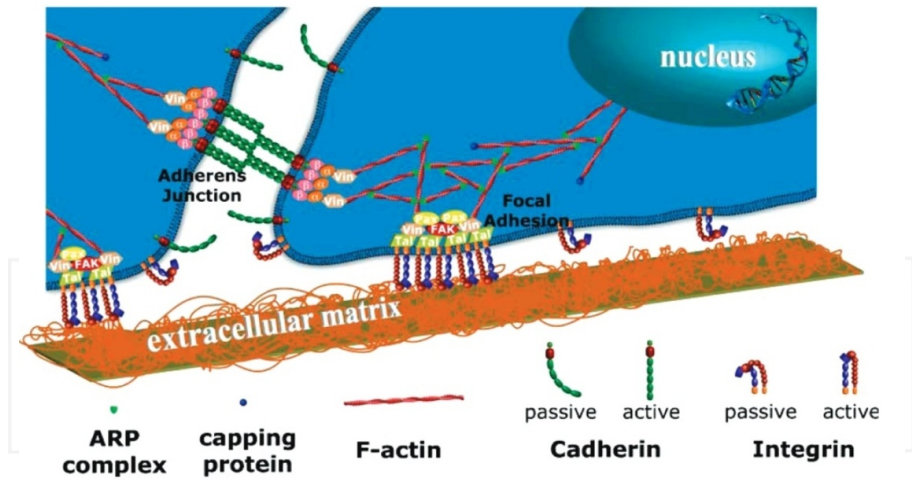

Figure 3. Schematic representation of cellular membrane, depicting different types of transmembrane proteins (open source).

2.2.1. What is ECM?

ECM is a protein-based support structure for cells, produced by cells. Initially, it was believed that it was relatively inert, but today it is known to interact intensely and specifically with cells. The main component categories of ECM are collagens (structural strength), elastin, proteoglycans (matrix resilience), and glycoproteins (cohesive bonding). These are quite general denominations for groups of proteins that show a surprisingly wide range of varieties, as it has been discovered in the past decade [8, 9]. Structure-wise, ECM is as varied as it is chemically diverse. In general, it could be described as a nanofiber matrix, mainly composed of collagen fibers, consisting of bundles of collagen fibrils that are anchored to the basement membrane, a dense sheet of collagen, lamilin, and other glycoproteins. How cells exactly interact with the ECM and how critical that interaction is, will be discussed next.

2.2.2. Cell–ECM communication

Cells sensing their environment will mostly limit themselves to contact-dependent communication pathways [8, 10, 11]. The main group of transmembrane proteins responsible for sensing and communicating with the ECM is the integrin family. Integrins are transmembrane

Figure 4. Schematic representation of integrin and cadherin activity [133].

glycoproteins consisting of α and β subunits with a large extracellular receptor domain that binds to certain RGD peptide sequences within the proteins (collagen, fibronectin, vitronectin...) that form up the ECM (see Figure 4). Up to this day about 20 different integrin transmembrane proteins have been discovered, each responding to different RGD peptide sequences. When an integrin binds onto the ECM, it will trigger the binding sites of the cytoplasmic tail, resulting in the formation of focal adhesion structures. These focal adhesions will bind to the cytoskeleton, triggering a cascade of intracellular signaling that will cause changes in the gene expression, which in turn affects all aspects of cellular behavior such as proliferation, differentiation, protein secretion, growth factor generation, and the generation of survival signals that prevent the cell to go into apoptosis. What makes cellular signaling (and communication) truly complex is that these transmembrane receptors rarely act alone: Most communication pathways consist out of multicomponent systems that depend on both the fixation of ligands on specific receptors as well as the transport of ions (e.g., Ca^{2+}) via ion channels [12]. Therefore, the key for designing a successful artificial matrix for the growth of a tissue is to simultaneously take into account multiple types of signaling molecules (immobilized on such artificial matrix), as well as their 3D distribution and density. In what follows next, an overview is given on how to design such 3D constructs.

2.3. Artificial ECM

The general idea behind tissue engineering is a three-step process (Figure 5): In the first step, healthy cells are extracted and isolated from the patient involved; In the second step, these cells are incubated in-vitro (in so-called bioreactors) on an artificial 3D construct that mimics the natural ECM [3–5, 13]; In the third step, after the formed tissue has reached a certain degree of maturation, the 3D construct is being implanted into the patient to replace the failing tissue

or organ. The material scientist within the group of tissue engineers is mainly interested in the artificial 3D construct used during the second step and how to optimize it in such a way that the seeded cells sense no difference compared to their natural support (ECM).
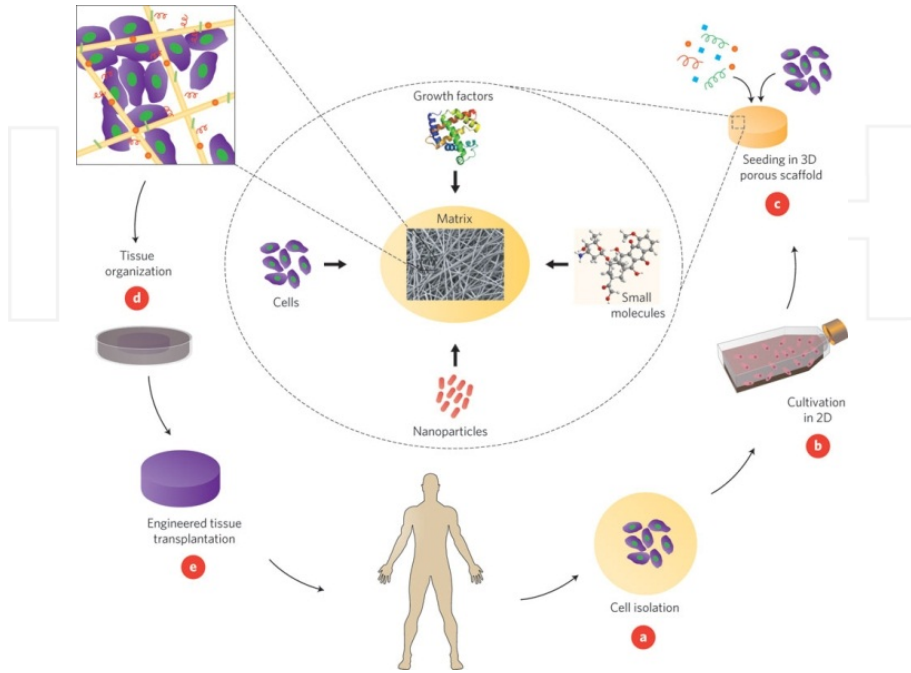

Figure 5. Schematic representation of the typical tissue engineering pathway followed [134].

2.4. Bio vs. artificial

The first step in fabricating appropriate cell support structures is the choice of material. As the support is implanted in-vivo and a second surgery has to be avoided, it is essential that the material is both biocompatible (preferably bioactive) and biodegradable [14–22]. This first condition is very generic, yet it rules out about 99 % of all materials available. Considering the remainder of the materials available, three different fabrication strategies can be followed: either work with a biopolymer that is sourced from a living organism (e.g., collagen from pigs), work with a synthetic material (e.g., polyesters or degradable metal alloys), or work with sheets of living cells as such. Each strategy has its advantages and disadvantages. Biopolymers such as collagen, gelatin, fibronectin... are all found in native tissue. Therefore, when cells are seeded onto such scaffolds, they do not lose their enzymatic activity to interact with and modify the surrounding ECM-like structure to their liking. The main downside using biopolymers is that they are extracted from foreign tissue, causing significant variability in quality as well as a constant risk for the transfer of

xenographic pathogens. From a mechanical point of view, most biopolymers do not have the structural strength to be used in any load-bearing applications, limiting themselves mostly to "soft" tissue applications. Most properties of synthetic materials are complementary to biopolymers, with their advantages pointing toward reproducibility, availability and price, degradation rate control, and mechanical strength. Their most critical downside is that their surface chemistry does not resemble the natural ECM at all. The interaction between the cells and their immediate environment will therefore be very different compared to their response to native ECM, thus preventing good communication between the cells and their surroundings, resulting in sub-optimal tissue generation. Cell sheets are a scaffold-free alternative but are quite difficult to grow and handling them without damaging the delicate sheet is not without challenge [18]. In all cases, there are solutions to the previously stated problems, some of them will be discussed further along in the chapter, and others fall outside of the scope of the chapter but are discussed in other excellent review papers [18, 20, 23–26].

2.5. (Bio)scaffold fabrication

Independent of the chosen material (living or dead), it still needs to be processed into a suitable 3D structure, resembling a natural extracellular environment. In the early days of in-vitro culturing, cells were seeded onto flat sheets and their response was studied. As scientists started to unravel the communication mechanisms of cells, it was concluded that 2D surfaces were bad models for cell studies, as cell morphology and behavior are greatly influenced by what it senses on all sides (see Figure 6). Alongside other developments in tissue engineering, material scientists and engineers therefore started developing techniques that would allow them to grow cell cultures in-vitro in a 3D environment [1, 27–29]. This research partially resulted in a new field within the tissue engineering community that is now known as biofabrication. In what follows, the most commonly used scaffold fabrication techniques will be discussed in more detail, followed by how non-thermal plasma technology can add value to the performance of such fabricated structures.

2.5.1. Conventional fabrication methods

Conventional methods for manufacturing scaffolds include solvent casting and particulate leaching, gas foaming, fiber meshes and fiber bonding, phase separation, melt molding, emulsion freeze drying, solution casting, and freeze drying [30].The repetitive limitations of this family of techniques is that they are all inherently limited when it comes to pore size control, pore geometry, pore interconnectivity, and the possibility to construct internal channels for transportation of oxygen and nutrients (see Figure 7). The limited poor interconnectivity prevents good cell migration, resulting in a high density of cells near the scaffold surface, with very little cells at the center.

Traditional scaffold fabrication methods were made very popular in the early days of tissue engineering, as the use of leaching salts, porogens, and supercritical fluids were cheap fabrication methods that did not require specific and/or expensive machinery [25]. To overcome the problem of poor pore interconnectivity, researchers at that time strived to develop
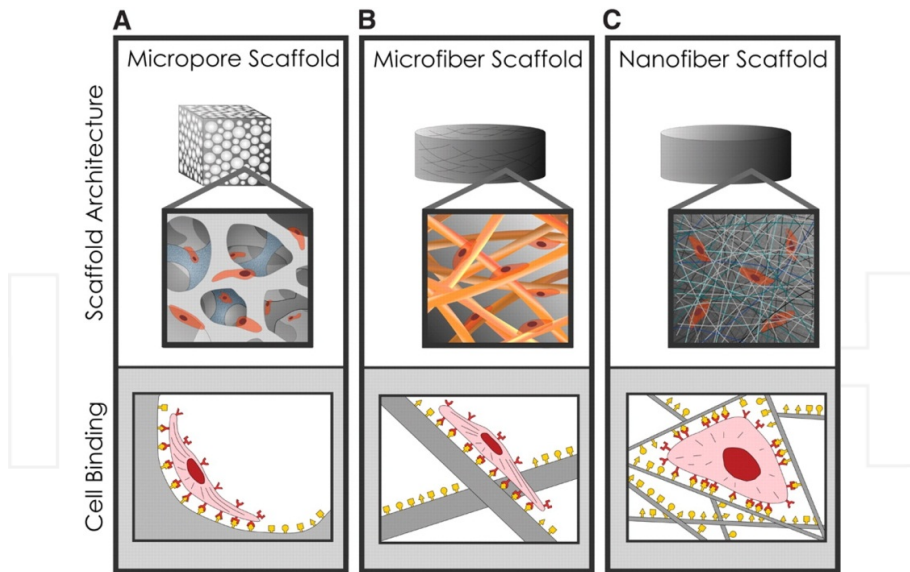
Figure 6. Differences in cell attachment efficiency between microporous and microfiber scaffolds on the one hand and nanofiber scaffolds on the other hand [135].

scaffolds with high pore ratios, up to 90 %. Although this partially resolved the pore inter-connectivity problem, it resulted in scaffolds with mediocre to poor mechanical properties [31].

A special case of a conventional scaffold fabrication method is the supercritical fluid process-ing. As it is a solvent-free technique, it is better suited than the other methods mentioned. Supercritical fluid (SCF) is created once a substance is exposed to an environment where its critical temperature and pressure are exceeded [32, 33]. A further increase in compression will therefore no longer result in liquefaction. Its physical properties are a combination of both liquid properties (density and solubility) and gas properties (diffusivity and viscosity). The most commonly used SCF is CO_2, as it is cheap, readily available, and reaches its supercritical point at near-room temperature (31°C, 7380 kPa). One of the more interesting properties of SCF's is their ability to dissolve polymers at a much lower temperature than their normal Tg. Making use of this property, Mooney et al. [6] were one of the first groups to apply this technique for the fabrication of microporous scaffolds. They dissolved a poly (lactic-co-glycolic) acid (PLGA) disc in CO_2 under SCF conditions. By rapidly decreasing the pressure, the solubility of the CO_2 decreases, causing nucleation and growth of gas bubbles within the polymer disc. The result is a sponge-like structure consisting of a high density of pores with varying size. The fact that no solvents were needed made it an excellent technique for tissue engineering applications. Several groups picked up on the technique, and it is still used to this day. Despite the lack of solvents during the production process, it suffers from the same limitations as the other conventional techniques though, inherently limiting it as a viable option for tissue engineering.
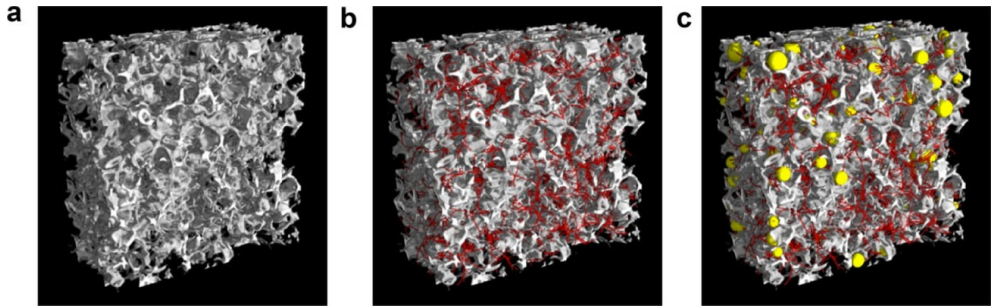
Figure 7. Micro-CT scan of traditionally fabricated scaffold via solvent casting/particulate leaching (A). B and C are showing the porous interconnectivity and pore size, respectively [136].

2.5.2. Rapid prototyping

Rapid prototyping is the collection of fabrication techniques that are able to directly translate a computer-generated design into a physical model. They are considered to be an additive process in which all parts of the construct are grown in a layer-by-layer fashion [20, 30, 34, 35]. The family of rapid prototyping techniques can be subdivided into two categories, depending on their mode of assembly: melt/dissolution deposition and particle bonding.

A melt/dissolution deposition system for tissue engineering applications can be considered as a miniature version of the traditional extrusion systems used in thermoplastic polymer processing, combined with an XYZ moving platform. The polymer, either molten or dissolved, gets forced through a small nozzle, after which it solidifies on the moving platform. Depending on nozzle size, temperature/concentration, and moving speed, a wide range of filament thicknesses can be extruded. Based on the digital design, a repetitive pattern can be printed, producing a porous structure with well-defined, well-interconnected pores in all directions, as can be seen in Figure 8. The typical pore size ranges typically from 100–1000 μm. More recently, the focus has shifted from thermoplastic biodegradable polymers to hydrogels, as several research groups have now succeeded to mix cells within the polymer feed [36, 37]. This generates hybrid scaffolds that are composed of both dead and living species.

Particle bonding systems use particles that are selectively bonded in a thin layer of powder material. These thin layers are then bonded onto each other in complex 3D structures, while being supported by unbounded material. At the end, the unreacted powder is removed and a well-defined interporous structure is obtained that exhibits both macropores and micropores (depending on the particle size of the powders). As the material is bonded via laser light or via adhesive droplets, dispersed via an inkjet type system, the amount of suitable polymers is limited.

While well-defined, geometrically complex 3D structures with excellent pore interconnectivity can be fabricated, they still exhibit a pore size that is an order of magnitude higher than what is found for natural ECM. This is not the case for that other popular scaffold fabrication technique: electrospinning.
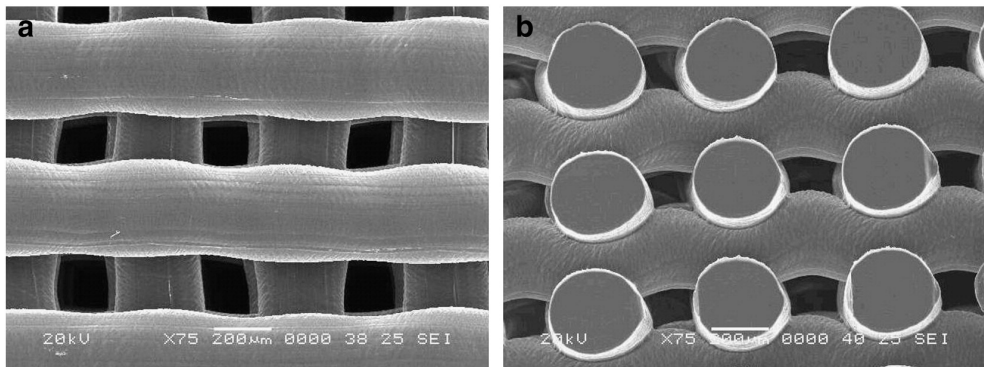
Figure 8. Top (A) and side (B) view of scaffold fabricated via microextrusion system [137].

2.5.3. Electrospinning

Electrospinning (ES) of polymers was already patented for the first time in 1939, but it was not until the early 1990 that a renewed interest in the 1D scaffold fabrication technique made it into the popular tool that it is today [38–43]. A typical ES machine consists of three major parts (see Figure 9): 1) a needle pump, feeding a polymer solution, at a certain flow, into the ES spinning chamber; 2) a high voltage source that is connected to the needle; 3) a grounded collector plate at a distance x from the needle. When applying a sufficiently high potential difference between the needle and the collector plate, the surface tension of the polymer droplet will break, resulting in the formation of a jet of polymer solution toward the collector plate. As the polymer solution travels at a high speed, the solvent will start to evaporate, resulting in the generation of nm-thick fibers that are then forming a randomly ordered 2D fiber sheet onto the collector plate (see Figure 9). The main difference between the ES and the other discussed scaffold fabrication techniques is the dimension range. As the fibers have a thickness in the order of nm, they are able to form μm- and nm-size pores, closely resembling the structures found in natural ECM. In the past 20–25 years, a wide variety of different polymers have been successfully electrospun, including most biodegradable and natural polymers. The biggest downsides of the ES technique are its lack in structural strength, thus preventing its use in load-bearing implant applications, and due to its dense packing, it makes it difficult for cells to migrate deeper into the spun matrix.

3. The added value of non-thermal plasma technology

As mentioned earlier, synthetic biodegradable polymers have a number of advantages (reproducibility, price...) and disadvantages over biopolymers and cell sheets [18]. Their main disadvantage is their lack of proper surface properties that stimulate the adhesion and proliferation of cells and differentiate them into the right type of tissue [44]. Numerous wet-
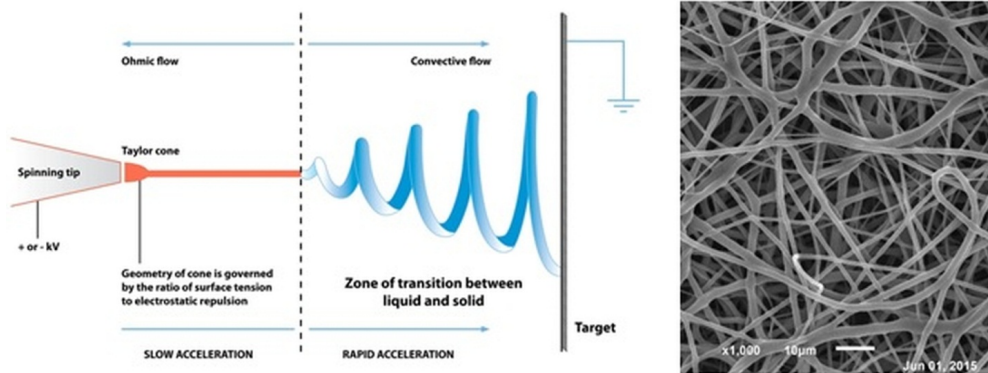
Figure 9. SEM micrograph of PCL electrospun fiber mesh (left: open source; right: original).

chemical techniques have been developed and applied, but most of them are either too aggressive, (partially) destroying the delicate structures, showing low treatment efficiency, time-consuming, or waste generating...

Non-thermal plasma technology is known as an excellent tool for surface engineering and a valid alternative for wet-chemistry-based surface engineering [14, 15, 45, 46]. It has been extensively used for surface decontamination/etching and surface treatment/activation on the one hand and the grafting/deposition of thin films with unique properties on the other hand. Its lack of solvents, fast treatment times, and preservation of bulk properties make it an ideal treatment method for delicate tissue engineered constructs [47]. Originally, research was focused on low-pressure plasma systems as they are well defined; exhibit excellent plasma stability; obtain good treatment efficiencies; and are able to deposit dense films that adhere well, are pinhole free, and show excellent stability. In the past two decades, there has been a shift toward research at (sub) atmospheric pressure as it eliminates the cost of extensive vacuum equipment and is less time-consuming, albeit not as well defined, and deposit films that are less dense.

In the following part of the chapter, an overview will be given on how both low-pressure and atmospheric pressure plasma technology have improved the performance of scaffolds for tissue engineering produced with different fabrication strategies.

3.1. Non-thermal plasma activation

Exposing a substrate to a plasma discharge fueled by an inert gas is the more basic approach for increasing the surface energy of said surface [14, 15, 48, 49]. Depending on the type of inert gas used, functional groups will be introduced directly (O_2, CO_2, air), indirectly (He, Ar) via post-oxidation processes, or a combination of both (N_2, NH_3, CF_4). At the same time, most plasma treatments will induce some degree of roughness, also contributing to the increase in wettability and surface energy, as has been extensively studied in the past on flat surfaces (see Figure 10). When applying a plasma treatment onto a scaffold structure, it is important to take

the time-dependent character of the treatment into account. The gradual decrease in treatment efficiency upon storage, also known as the aging effect, is something that is often overlooked in literature. The substrate surface will try to return to a more stable energy state by lowering its surface energy again. This is either accomplished via polymer chain reorientation or the desorption of low molecular weight components. Depending on the substrate characteristics (crystallinity, molecular weight, storage atmosphere), they will lose up to 90 % in treatment efficiency over a period of only weeks. Using proper storing conditions (vacuum, low temperature) can slow down the ageing effect significantly, though it is something that has to be kept in mind when developing scaffolds for tissue engineering applications.
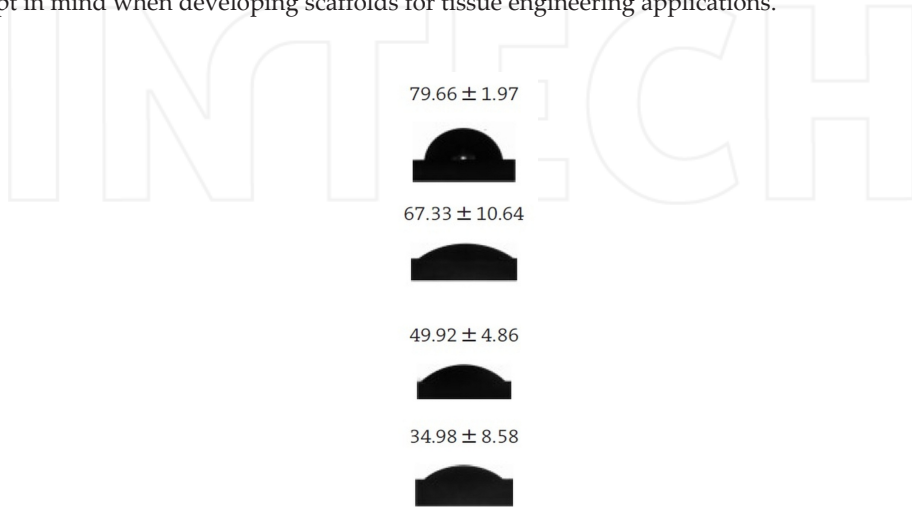
Figure 10. The change in WCA after 5 min of air plasma treatment at atmospheric pressure [129].

In the overview of the literature, it is clear that the developed non-thermal plasma surface modification strategies were in their early stages. As the scaffold fabrication methods evolved, plasma technological solutions also became more in tune with their microbiological environment. This specific chapter section therefore consists mainly out of basic surface-cell interactions, with the more advanced modification strategies following thereafter [138].

3.1.1. Traditional fabrication methods

To our best knowledge, it was not until 2002 that the first articles were published on the treatment of traditionally produced scaffolds with non-thermal plasma. Chim et al. [50] used a vibrating salt leaching method to fabricate poly(D,L) lactic acid (PDLLA) scaffolds, after which they applied an oxygen low-pressure radio frequent (RF) plasma (100 W–3 min). Static water contact angle goniometry (WCA) measurements showed a decrease in contact angle from 80° down to 60°, indicating an increase in surface polar groups, yet no X-ray photoelectron spectroscopy (XPS) or secondary ion mass spectrometry (SIMS) was performed to quantify this. Scanning electron microscopy (SEM) measurements revealed an increase in surface roughness as well as the formation of sub-µm-sized pits. The seeding of embryonic palatal

mesenchyme (HEPM) cells into the scaffold resulted in a five-fold increase in cell activity compared to untreated scaffolds, while measurements of alkaline phosphatase activity (ALP) indicated an accelerated cellular differentiation into osteoblasts. Köse et al. [51] followed a similar strategy for the fabrication and treatment of poly(3-hydroxybutyrate-co-3hydroxyvalerate) (PHVB) scaffolds and their subsequent seeding with osteoblasts.

Around the same time, Claase et al. [52] used both freeze drying and salt leaching to synthesize scaffolds from different poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) mixtures, a copolymer that supposedly would enhance osseointegration. First lab tests revealed no significant increase in osseointegration, independent of composition. A CO_2 RF low-pressure plasma (49 W–30 min) was applied, but no surface characterization techniques were used to analyze its effects. Goat bone marrow cells were seeded into the scaffolds, after which a whole range of in-vitro tests revealed that the plasma treatment had a positive effect on adhesion and proliferation, but only for certain copolymer compositions (PEOT/PBT in ratio 70/30), while pore size was only of minor influence. A few years later, the experiment was repeated with rat bone narrow stromal cells, giving very similar results for the growth of cartilage rather than bone [31].

It is now considered to be general knowledge that surfaces rich in primary amines are excellent for the initial adsorption of certain proteins, thus positively stimulating cell behavior. Yang et al. [53] were, to our knowledge, the first to use an ammonia low-pressure RF plasma (50 W–5 min) on traditionally fabricated scaffolds (solvent casting + salt leaching specifically). WCA measurements on the plasma-treated poly-L-lactic acid (PLLA) and PLGA scaffolds indicated a high increase in wettability (78° → 15°), while XPS showed the incorporation of 4–6 % of nitrogen. Human foreskin fibroblasts were seeded, resulting in a significant increase in cell viability according to the performed MTT assays. Fluorescence microscopy showed an elongated morphology and a distinctively higher seeding efficiency.

Choi et al. [54] focused on the surface alteration of commercially available calcium phosphate scaffolds (200–400 μm pore size) using an atmospheric pressure plasma jet (APPJ) with either air or nitrogen as a discharge gas (15 kV, 13 mA, 5 slm, 10–20 min). WCA were reduced from 80° to <10°. XPS analysis revealed that the relatively high hydrophobicity was caused by significant carbon surface contamination, which was sharply reduced by the plasma treatment in favor of the introduction of –OH groups onto the surface. MC3T3 mouse osteoblasts were seeded on scaffolds treated with either air or nitrogen. Adhesion assays revealed a significant increase in cell adhesion compared to untreated scaffolds, even though no differences were found between treatments. Proliferation analysis revealed though that cells seeded onto nitrogen-treated surfaces proliferated at a higher rate, which can be linked back to the influence of amine rich surfaces. Morphology analysis learned that cells were still quite round, suggesting that the activation step as such is insufficient. This indicates that a grafting step or coating step might be better for initial cell-surface interactions, while still benefiting from the long-term effects of the underlying CaP substrate.

In the following 13 years, several other research groups have performed comparable plasma surface treatments on scaffolds produced via conventional fabrication methods. Their results have been summarized in Table 1. Most found significantly better cell adhesion and good cell

proliferation. Ring et al. [55] also proved that a plasma treatment can lead to better neovascularization, while Shah et al. [56] showed that the results obtained in-vitro do not always translate well in-vivo, bringing a whole new set of challenges.

| Author	year	scaffold type	fabrication method	reactor & gas	cell-type	effect
Safinia et al. [57]	2005	PDLLA	thermally induced phase separation	low pressure RF air &NH$_3$	/	higher wettability
Wan et al. [58]	2005	PLLA	phase separation	low pressure RF NH$_3$	M3T3 fibroblasts	better cell proliferation
Safinia et al. [59]	2007	PS/PLGA	thermally induced phase separation	APPJ	/	higher wettability
Ring et al. [55]	2010	Matriderm®	commercial	low pressure RF Ar/H$_2$	in-vivo	better neovascularization
Han et al. [60]	2011	PLA-PCL	solvent casting/ salt leaching	atmospheric pressure bipolar DC	L929 & MC3T3 osteoblasts	no cytotoxicity & homogeneous distribution through scaffold
Shah et al. [56]	2014	PLA	vibrating particle/ salt leaching	low pressure RF O$_2$	osteoblast & endothelial co-culture	in-vitro synergetic in-vivo location dependent
Bak et al. [61]	2014	PCL	gas foaming/ salt leaching	low pressure RF O$_2$ & N$_2$	MC3T3 osteoblasts	enhanced adhesion and proliferation
Sardella et al. [62]	2015	PCL	solvent casting/ salt leaching	low pressure RF H$_2$O/N$_2$	Saos-2 osteoblasts	50/50 H$_2$O/N$_2$ best cell adhesion and proliferation
Trizio et al. [63]	2015	PCL	solvent casting/ salt leaching	APPJ He/O$_2$	Saos-2 osteoblasts	cell-clustering, polymorphal shape slow spread throughout scaffold

Table 1. Overview of literature on plasma modification of traditionally fabricated scaffolds not discussed in the text.

3.1.2. Rapid prototyping

Rapid prototyping techniques were introduced to the tissue engineering community in the late 1990s [64–66]. Still, it took almost a decade before the first papers were published that involved plasma treatment of such structures, with the majority of the papers published in the past 5 years. The first paper, to our knowledge, was published by Wagner et al. in 2006 [67]. They developed an in-house system and printed PLGA scaffolds with 1.3 mm (!) gaps, after which they treated them with an oxygen low-pressure RF plasma (0.04 kPa, 5 min, 15 kV). No surface analysis was performed. Ovine and human osteoblasts were seeded, and results were compared to untreated scaffolds and tissue foil. Improvements in cell adhesion and prolifer-ation were found compared to the untreated scaffolds, but results were worse compared to the tissue foil. In theory, one can still talk about a 3D scaffold in this case but with such pore sizes results will always be similar to what is found for cell-seeding on 2D films, as the gaps will be too big for cells to bridge.

In 2007, Moroni et al. [68] applied an Ar low-pressure RF plasma treatment (0.01 kPa, 30 min) on PEOT/PBT scaffolds printed on an Envisiontec® system. Both scaffolds based on generic designs as well as those based on CT scans from actual patients were used in this study. Again no surface analysis was performed. A SEM study of the scaffolds revealed a significantly higher degree of printing imperfections for the patient-based scaffolds. Yet, when in-vitro tests (MTT + GAG) were performed, the patient-based scaffolds performed remarkably better in terms of tissue formation and differentiation compared to the generic ones, both being treated with the non-thermal plasma. This indicates that biomimetic scaffold designs also play an important role for successful tissue engineered constructs.

Yildirim et al. published two papers on the modification of polycaprolactone (PCL) scaffolds with pore size 300 μm, printed using an in-house system [69, 70]. In both cases, an O_2 low-pressure RF plasma (18 W, 0–7 min) was applied. WCA measurements showed a progressive increase in wettability (58° → 22°). XPS measurements revealed an initial increase in oxygen functional group incorporation (up to 1 min treatment). Further treatment resulted in a decrease to values similar to untreated PCL. This suggests that the main effect of the oxygen plasma is etching rather than functional group incorporation. 7F2 mouse osteoblasts were seeded in both cases, giving similar results: enhanced adhesion and differentiation (higher levels of ALP and osteocalcin), and accelerated formation of mixed mineralization, all indica-tions of increased osseointegrating properties. In 2012, Jacobs et al. [71] performed a more systematic study of different plasma discharge gasses (Ar, He and air) on PCL printed scaffolds, using a medium pressure parallel-plate system (5 kPa, 1–2 W, 30–300 s). Short treatment times resulted in the formation of a wettability gradient toward the center of the scaffold, as visualized by a standardized ink-staining method [72]. Longer treatment times resulted in a homogeneous wettability throughout the scaffolds, as confirmed by XPS (%O + 7 %). MC3T3 osteoblasts were seeded into the scaffold. Initial cell adhesion, as well as cell proliferation, greatly increased for those scaffolds treated by plasma, covering the complete interior of the scaffold (see Figure 11). Cell morphology was more elongated, and protein levels were higher.
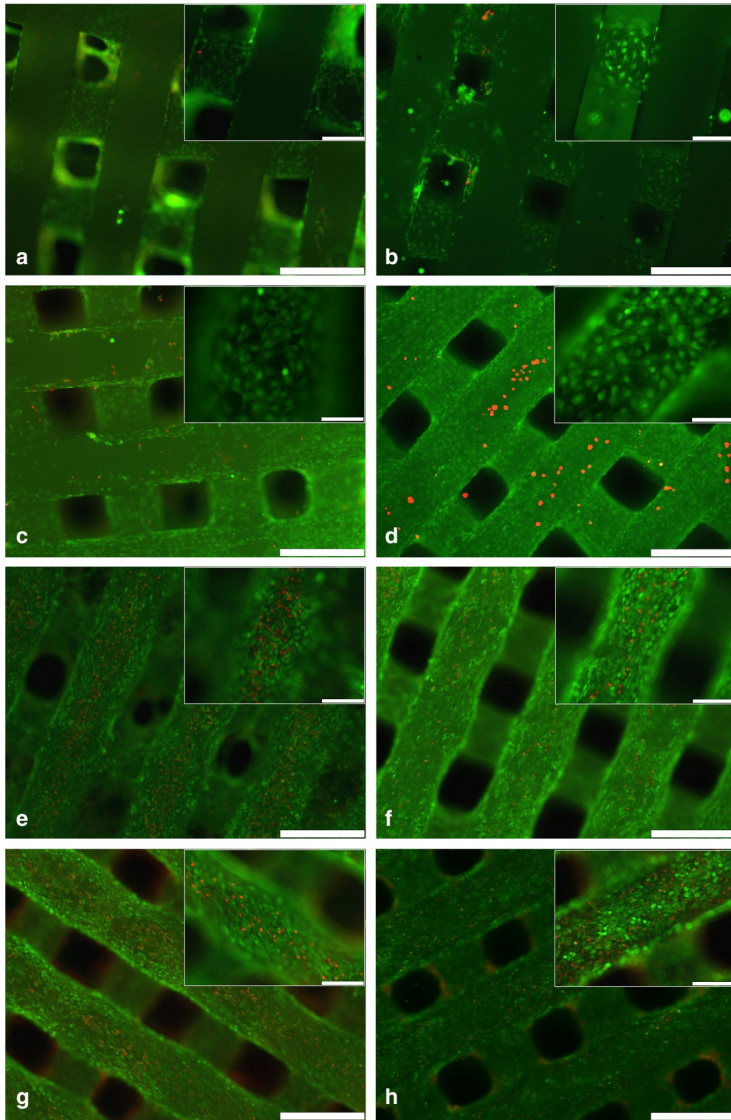
Figure 11. Fluorescent micrographs of PCL scaffold cross-sections of MC3T3 seeded osteoblasts. Left, from top to bottom (a–e): untreated scaffolds after 1–3–7–21 days of seeding. Right, from top to bottom (b–h): air plasma-treated scaffolds after 1–3–21 days of seeding [137].

3.1.3. Electrospinning

To this day, electrospun or e-spun fibers are considered to be the closest (semi)synthetic alternative to natural ECM, being one of the only techniques that is able to reproducibly

fabricate scaffolds with sub-μm features on a larger, economically viable scale. As already mentioned, it is a relatively old technique (1939) that only gained renewed interest two decades ago, it was not until 2005 that the first papers were written on the plasma modification of such fibers.

Baker et al. [73] were one of the first groups searching for a non-invasive method to improve the cell attachment of finite smooth muscle cells on both randomly and aligned polystyrene (PS) fibers. An Ar RF plasma was applied (296 W, 0.01 kPa) for 5 min, and surface analysis was performed. The speed of adsorption of water was so high that it was impossible to measure correct WCA, while XPS measurements revealed a 20 % increase of oxygen, which is similar to results found for 2D films [74]. Cell attachments assays indicated a twofold increase in adhesion and actin staining images show that the cells align very well along the direction of the fibers. Despite the excellent in-vitro results, serious questions have to be raised to the use of PS as a modern implant material, as it is not biodegradable.
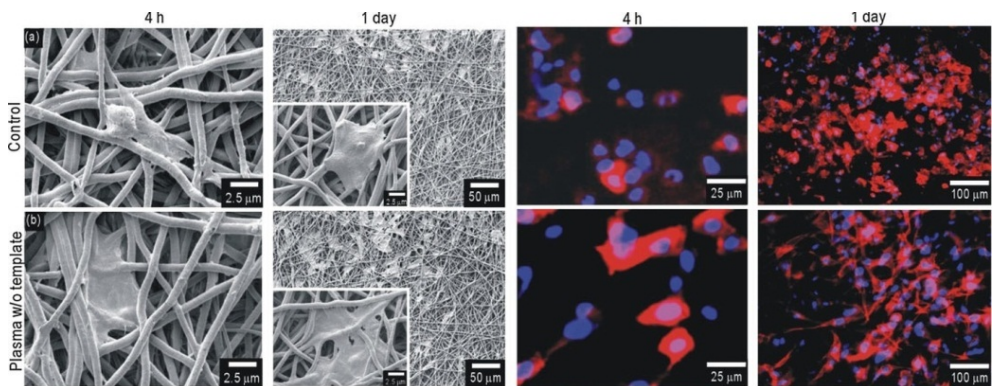
Figure 12. Improved cell infiltration into electrospun scaffolds after plasma treatment (left) and improved cell morphology (right) Jeon et al. [139].

Synthetic biodegradable polyesters such as PLA and PCL were often the material of choice for the fabrication of traditional and rapid prototyped scaffolds, and this is no different for the fabrication of electrospun materials. Several publications were published in the last 7 years where either PCL or PLA nanofibers were exposed to a plasma treatment [75–84]. Both low-pressure RF systems and atmospheric pressure plasma jets (APPJs) were used to generate plasma discharges using a variety of discharge gasses (air, NH_3, Ar, $N_2 + H_2$, O_2, or a combination of those). As was the case for the other fabrication techniques, several publications lack proper surface analysis, hence introducing a novel surface modification technique, but not characterizing what changes are taking place and to what extent. Those papers that did include a (partial) material analysis found the following results: WCA for PLGA fibers were decreased from 135° to around 45° in most cases. For PCL and PLLA, most were able to achieve full adsorption of the droplet (WCA =0°). XPS measurements showed an increase in oxygen containing functional groups between 2 and 7 % for PL(G)A, pure O_2 plasma treatment being

the only one to achieve an increase of more than 4 %. The oxygen content of PCL fibers was increased up to 12 %. Those papers that used N_2 or NH_3 were able to incorporate up to 5 % in nitrogen containing groups, but no additional derivatization of alternative techniques were used to see what type of nitrogen groups were incorporated onto the surface. The changes in surface wettability came at a cost though. Tensile tests revealed a reduction between 30 and 40 % in tensile strength, indicating that plasma no longer can be viewed as a non-invasive technique when it comes to scaffolds with sub-μm dimensions. Whereas the papers in most cases lack proper surface characterization, they excel in providing good quality in-vitro and in-vivo results. Almost all studies revealed an increase in initial adhesion and cell morphology and this for practically any cell-type tested (fibroblasts, osteoblasts, smooth muscle cells, stem cells, Schwann cells…), but some studies went into even more detail. Park et al. [78] studied the adsorption of Bcl-2 proteins as a function of incorporated N-density on their e-spun PLGA scaffolds. Results showed that a mediocre hydrophilicity (WCA between 50° and 60°) resulted in the highest uptake of said protein. In parallel, they seeded fibroblasts onto the ammonia-treated scaffolds and found that the protein expression of the cells followed the same trend, with an overall reduction in the expression of stress-induced reactive oxygen species (ROS) secretion. De Valence et al. [79] tested their air plasma-activated PCL vascular grafts in-vivo, even after disappointing results in-vitro. Whereas the smooth muscle cells showed no significant increase in proliferation in-vitro, in-vivo tests revealed that cells were able to penetrate the scaffolds more efficiently and at higher densities, mainly due to the increased wettability. Cheng et al. [85] found similar results in-vivo for their Ar-/NH_3-treated PLA scaffolds – indicating that for cell infiltration, wettability is most likely the critical factor. When it comes to mineralization, Yang et al. [76] found similar results compared to Yildirim et al. [86] in the rapid prototyping section: improved mineralization within the first few hours after immersion of Ar plasma-treated scaffolds into simulated body fluid (SBF) solution. This resulted in the complete filling of the (sub)μm pores within 6 hours. After 7 days, the CaP was converted into type B carbonate apatite, the same building block found in natural apatite. Still, due the inferior properties of e-spun fibers compared to printed structures, it is to our belief that e-spun scaffolds as such will never be used for the repair of bone-related defects, unless combined with a polymeric, metallic, or ceramic support structure.

Whereas most other scaffold fabrication techniques were limited to biodegradable polyesters, electrospinning has a much broader spectrum of materials available. Biodegradable polyur-ethanes (PU) are a viable alternative to biodegradable polyesters, yet it is known that in some cases they trigger a more aggressive immunological response upon implantation [87]. Zandén et al. [88, 89] published two papers on the influence of O_2 RF low-pressure plasma treatment on the haemocompatibility of PU meshes and their influence on human embryonic stem cells. In both papers, they had to conclude that a plasma treatment as such is insufficient to signif-icantly alter the haemocompatibility or to stimulate the differentiation of stem cells. Ardeshir-ylajimi et al. [90] exposed polyether sulfone scaffolds (PES) to an O_2 low-pressure microwave (MW) plasma (0.04 kPa, 10 min). In-vivo, studies with rabbits revealed that the scaffolds induced an increase in ALP levels and a significant increase in calcium content was found. The relative expressions of Runx2, Col 1, osteonectin, and osteocalcin analyzed via reverse transferase–polymerase chain reaction (RT-PCR) were all elevated within the first week.

Surprisingly, analysis of scaffolds implanted for more than 7 days showed that the expression levels dropped drastically and no more significant differences were found compared to untreated samples (which might be due to possible ageing effects of the plasma-treated scaffolds).

Electrospinning is the only scaffold fabrication technique, to our knowledge, where plasma has been successfully applied to improve the surface properties of biopolymers. In general, biopolymers are frequently used to fabricate scaffolds via other scaffolding techniques (e.g., rapid prototyping), but due to their high water content, it is less obvious to apply a non-thermal plasma, whereas electrospun materials are normally used in a dehydrated state. Baek et al. used an MW induced Ar plasma jet to modify electrospun/salt leached silk fibroin scaffolds (400 μm) intended for cartilage repair [91, 92]. Neonatal human knee articular chondrocytes were seeded onto scaffolds, resulting in a 50 % increase of initial cell attachment and a 100 % increase of proliferation compared to untreated scaffolds. Although, when the GAG content was analyzed, little to no difference was found for the treated scaffolds, indicating yet again that plasma treatment is not specific enough when it comes to the stimulation of secondary processes such as the enzymatic excretion of polysaccharides.

3.1.4. Intermediate conclusion

The overall conclusion that can be made for plasma-treated scaffolds is that it greatly increases the wettability of the scaffolds, allowing for a more efficient seeding process. This is reflected in significantly higher cell adhesion within the first 24 hours and a better distribution of the cells throughout the scaffold. After 7 days, though, the advantages of plasma treatments on cell proliferation are in most cases less substantial. This is most probably due to the non-specific nature of the introduced functional groups onto the surface of the scaffold interior. In the introduction, it was mentioned that cell signaling is highly dependent on the type and distribution density of transmembrane proteins protruding from the cell surface. Both the type of substrate functional groups and their density thus play a major role in the metabolic pathways of the cell. A more hydrophilic surface does allow cells to initially attach better, but as the correct integrin bond is missing, proper cell proliferation, and differentiation are inhibited. Nitrogen-rich surfaces perform better compared to oxygen rich surfaces, as in most cases they will contain a certain amount of primary amines, which is one of those functional groups preferred by cells for bone regeneration (or by the proteins initially excreted by the cells or present in the growth medium). However, there is often no control of functional group densities. Therefore, to better mimic the ECM environment, it would be recommended to have a higher control over the type of functional groups on the surface and their distribution, which are two parameters that can be better controlled by plasma grafting and plasma polymerization, which will be discussed next.

3.2. Plasma grafting and polymerization

Thin polymer-like films deposited via plasma polymerization are considered very different from films deposited via traditional wet-chemical techniques in the sense that the monomer gets fractionalized when exposed to plasma. This results in highly branched, highly cross-

linked amorphous networks. These nm-thick films are often characterized as highly stable, dense, extremely adhesive to the substrate, and pinhole free (although not all these characteristics transfer well from low-pressure systems to atmospheric pressure systems and are influenced substantially by the set of plasma parameters used). As it is a solvent-free deposition technique, it is considered an excellent tool for tissue engineering applications.

Plasma grafting is a hybrid technique positioning itself between plasma treatment and plasma polymerization. The plasma step itself is identical to a plasma treatment, the (macro)monomer being introduced only after plasma treatment. Either the introduced reactive sites are then used directly as initiation points for free radical vinyl polymerization or afterwards by using the introduced oxygen/nitrogen functional groups to covalently bond other (macro)molecules, involving a wet-chemical step. This results in coatings with excellent retention of the functional groups, but low surface functional group density control. Both techniques are considered superior compared to plasma treatments when it comes to the variety of functional groups that can be introduced onto a scaffold surface and their stability over time.

3.2.1. Traditional fabrication methods

One of the best-known papers on plasma grafting and plasma polymerization applied on traditionally fabricated PDLLA scaffolds (supercritical CO_2) was published in 2005 by Barry et al. [93]. In this paper, an O_2 low-pressure RF plasma was used (40 Pa, 3–20 W, 3 min) to compare the penetration efficiency of allylamine when plasma grafted or plasma polymerized, respectively. XPS cross-sectional analysis revealed that for the grafting an evenly distributed nitrogen signal could be found, but at relatively low atomic concentrations (2 %) (see Figure 13). Plasma polymerized allylamine resulted in incorporation of up to 10 % nitrogen but after washing a parabolic gradient was found. They linked this gradient to limitations in diffusion of the allylamine precursor due to the limited pore interconnectivity. For the grafting step, as they allow the monomer to flow through the scaffold for up to 10 min, they claim that it eventually will penetrate all of the scaffold, but at a lower incorporation efficiency. In-vitro tests, using M3T3 fibroblasts revealed that only for the plasma polymerized scaffolds the cells were able to penetrate to the center of the scaffolds, while at the same time showing higher densities a the scaffold surface. This indicates that a certain density of polar functional groups is required to allow cells to effectively migrate throughout the scaffolds. Furthermore, it is an important indication of the diffusion limitations of traditionally fabricated scaffolds: Allylamine is a very small molecule, yet sharp gradients in coating chemical composition could be found. The diffusion limitations will increase exponentially when applying larger precursors for polymerization or trying to immobilize larger biomacromolecules such as collagen, gelatin, fibronectin...

Six years later, this research was picked up again by the same group in collaboration with the University of Bari, resulting in the publication of two papers by Intranuovo et al. [94, 95]. In the first paper, published in 2011, a very similar experiment was conducted involving low-pressure RF pulsed plasma polymerization of allylamine, with very similar results when it comes to gradient deposition. To improve the migration of the 3T3 fibroblasts used in the experiment, a hexane plasma was applied for a very short time (30 s) directly after the
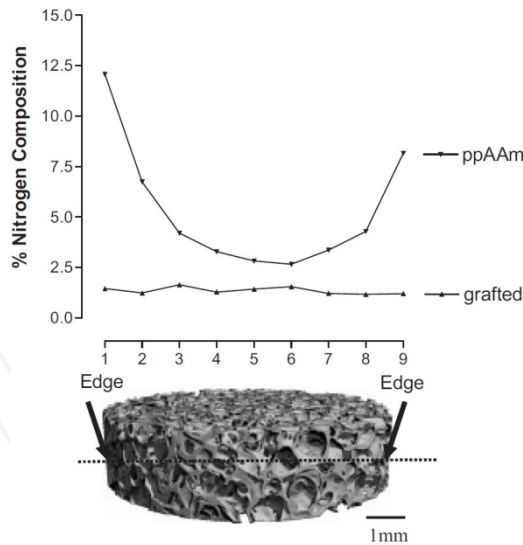
Figure 13. Distribution of nitrogen through traditionally fabricated scaffold: plasma grafting versus plasma polymerization [140].

allylamine plasma polymerization step. Hexane, being a bigger molecule, was limited even more when trying to diffuse into the scaffold. Combined with the short treatment time, this resulted in a hydrophobic outer surface of the scaffold. Upon seeding, the fibroblasts were "forced" to migrate into the scaffold. 96 hours after seeding cross-sectional imaging revealed a homogeneous distribution throughout the scaffold. In the paper published 3 years later, they again repeated the experiment, but using C_2H_4/N_2 mixture (3 min) instead, followed by a hydrogen plasma (30 s) to introduce primary amines onto the surface. In a second step, they solely used C_2H_4 plasma (90 s) to introduce reverse gradients (more nitrogen in the central part of the scaffold). Having used even smaller molecules compared to allylamine paid off, as the gradient throughout the scaffolds was far less pronounced, with up to 7 % of nitrogen functional groups in the center of the scaffolds. This also showed in the in-vitro testing, with Saos-2 osteoblasts evenly distributed for scaffolds both with and without the ethylene plasma step, thus being an example of an over-engineered solution.

In two papers published by the biomaterials research center of South Korea, O_2 low-pressure RF plasma systems (30 s, 0.1 Pa) were used to activate PLLA scaffolds, followed by an acrylic acid grafting step [96, 97]. Chondrocytes were seeded onto the scaffolds, and significant increase in adhesion and proliferation was found. Four weeks after seeding, higher levels of collagen II were measured as well as increased GAG levels, indicating the formation of cartilage. The success of the in-vitro tests was related to the acid rich surface, as well as an increase in surface roughness. Demirbilek et al. [98] focused their study on the changes in oxidative stress caused by plasma grafting. They used the freeze drying technique to fabricate their scaffolds. After plasma treatment (no details given), scaffolds were grafted with either

ethylene diamine or low-molecular weight PEG. L929 osteoblasts were seeded, and oxidative stress was measured both via malondialdehyde and advanced oxidation protein products. Surprisingly, results showed that the level of oxidative stress was greatly reduced compared to cells grown on untreated scaffolds, even for PEG-grafted scaffolds, which are normally considered to be anti-fouling.

Several authors used low pressure plasma systems to activate PLGA scaffolds (O_2/CO_2, Ar and NH_3 plasma, respectively) fabricated via phase separation/ salt leaching in order to immobilize a biomacromolecule of choice [99–101]. Hu et al. [101] immobilized both polylysine (enhances electrostatic interactions) and RGD peptides, using a glutaraldehyde linker. Osteogenic precursor cells were seeded onto the scaffolds and after 14 and 28 days, alkaline phosphatase (ALP) activity and calcium assays were performed to determine the level of osteogenic differentiation. Results showed that the introduction of charge (either via NH_2 or polylysine) was not enough to stimulate differentiation, whereas the introduction of RGD peptides resulted in a significant increase in ALP activity and calcium concentration. This indicates that the RGD-peptide activity is well preserved, resulting in a stimulated osteogenic differentiation of the precursor cells. Woo et al. [100] immobilized β-glucans, a polysaccharide known for its enhanced wound healing properties, onto their scaffolds. Adipose tissue-derived stem cells were seeded onto the scaffolds, resulting in enhanced cell proliferation and improved cell morphology (more spindle like). Unfortunately, no studies were performed on differentiation. Shen et al. [99] immobilized rhBMP-2, an osteoinductive protein. Different plasma discharges were tested, and those that introduced a negatively charged surface were found to be most efficient in immobilizing the positively charged protein (O_2 and CO_2 vs. NH_3). OCT1 osteoblasts were seeded and ALP activity measurements indicated a significant increase, while SEM images showed accelerated mineralization within 22 days. Zheng et al. [102] used a different approach to obtain similar results, using a PLGA-tricalciumphosphate composite scaffold (phase separation + particle leaching). After ammonia plasma treatment, the scaffolds were dipped in a collagen solution, effectively immobilizing some of it onto the surface. Ex-vivo analysis of seeded rat dental pulp stem cells indicated greatly enhanced ALP activity (20 times higher), while von Koss staining showed increased areas of mineralization and Azan staining showed the presence of dentin proteins, all of which are indications of dentin-like bone formation.

Overall, quite a diverse group of traditional scaffold modifications has been discussed above. Compared to the plasma treatment sections, results are far more promising when it comes to cell proliferation, and especially cell differentiation. In-vitro, ex-vivo, and in-vivo experiments have shown that plasma-modified traditional scaffolds could be successfully applied to grow bone and cartilage tissue. Most papers though, avoid the topic of treatment homogeneity, while the first few papers discussed in this section, as well as papers discussed in the plasma treatment section, clearly show that this is an important issue to take into account. With this gradient problem taken into account, it is no surprise that there are very few papers available that focus on plasma polymerization of traditionally fabricated scaffolds or on the distribution homogeneity of the larger immobilized biomolecules such as rhBMP-2, β-glucans, or collagen. For both rapid prototyped structures, with their high pore interconnectivities, and electrospun fibers, with their extremely high porosity, this should not be such an issue.

3.2.2. Rapid prototyping

All papers found on plasma grafting and polymerization for rapid prototyped scaffolds were published in the last 4 years, indicating a large potential for further studies. Between 2012 and 2014, several papers were published by Declerq et al. and Berneel et al., following the same plasma grafting procedure [103–105]. PCL scaffolds (200 μm pore size) printed via a bioscaffolder (SYS + ENG) were exposed to an Ar DBD plasma for 30 s, followed by immersion in a 1M 2-aminoethylmetacrylate solution and subsequent exposure to UV radiation for 60 min. In a consecutive step, gelatin B (GelB) was immobilized on the introduced primary amines via carbodiimide wet chemistry, and finally, those samples were immersed in a fibronectin solution, resulting in an active physisorption of the protein (6 ng/mm²). In the initial study published in 2012, both HFFs and MC3T3 osteoblasts were seeded on the scaffolds and the improvements on cell colonization for each consecutive coating step (plasma treatment → AEMA → GelB → fibronectin) were studied. Cross-sectional images 21 days after seeding show that the osteoblasts only grow on the edges of the plasma-treated samples, while for all other samples, they proliferated and migrated completely into the scaffolds (see Figure 14). For the fibronectin rich samples, the highest density of cells was found, including the formation of new natural ECM. In a second study, the performance of adipose-derived stem cells on fibronectin rich scaffolds was compared to commercially available collagen hydrogel scaffolds. Initially, the collagen scaffolds performed significantly better for seeding efficiency (×2) and protein production (+50 %) compared to the fibronectin rich scaffolds, which can most likely be contributed to the design of the scaffolds rather than the surface chemical properties. After 28 days, RT-PCR was performed to analyze the differentiation efficiency and selectivity. Results show that, although there was a delay in differentiation compared to the more compact collagen scaffolds, the fibronectin scaffolds show a better and more stimulated osteogenic differentiation compared to the commercial collagen scaffolds. Further improvement of scaffold designs, combined with higher concentrations of immobilized fibronectin should lead, in our opinion, to higher seeding efficiencies and even more pronounced differentiation. Van Bael et al. [106] repeated the exact same procedure on PCL scaffolds fabricated via selective laser sintering (SLS) without the fibronectin physisorption and found very similar results.

Sousa et al. [107] used the BioExtruder to fabricate PCL scaffolds with pore size diameters of 900 μm. An Ar low-pressure RF plasma (100 W, 30 s) was applied and exposed to ambient air to allow the formation of peroxides and hydroxyperoxides on the scaffold surface. This was followed by an immersion in an acrylic acid solution combined with exposure to UV-irradiation. In a final step, collagen was immobilized onto the surface using carbodiimide wet chemistry. M3T3 fibroblasts were seeded onto the surface, and MTT tests were performed after 7 and 14 days. Results show no significant differences toward the untreated scaffolds, but more importantly, also no significant differences were found compared to commercially available collagen scaffolds, unlike to what was found by other researchers. This is a strong indication that the type of cell used for seeding (fibroblast vs osteoblast vs. stem cell) as well as the origin of the cell (primary cell lines versus cancer cell lines) and scaffold design all play an important role on the possible in-vitro outcome of the study.
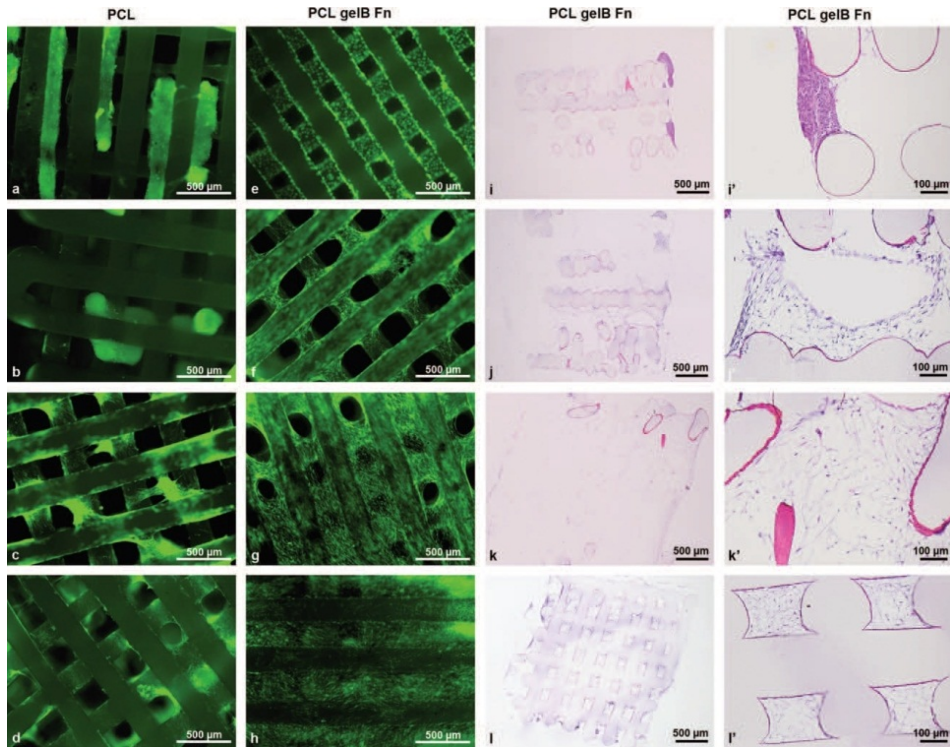
Figure 14. Viability, infiltration, and colonization on PCL and PCL GelB Fn scaffolds. Calcein AM images of HFF cells after 1, 7, 14, and 21 days on PCL scaffolds (a–d) and PCL GelB Fn scaffolds (e–h). Colonization of the 3D scaffolds by HFF cells 1, 7, 14, and 21 days post-seeding. Histological sections taken of the center of PCL GelB Fn scaffold [scale bar 500 lm (i–l)]. Details of the histological sections of the center of PCL GelB Fn scaffolds are given in i'–l' (scale bar 100 lm). Hematoxylin/eosin staining of 5-lm thick paraffin sections. The pink lining is representative for the gelatin coating. Only when cells were present, the integrity of the scaffolds could be preserved [142]

Nebe et al. [108] also used SLS to fabricate PLGA/tricalcium phosphate composite scaffolds with a 500-µm pore size. An O_2 low-pressure MW plasma was applied (500 W, 5 Pa, 0.1 slm, 10 s) followed by a plasma polymerization step using allylamine as a precursor (500 W, 50 Pa, 0.05 slm, 144 s), resulting in the incorporation of up to 16 % of nitrogen, as confirmed via XPS (no mention of cross-sectional analysis). MG-63 osteoblasts were seeded onto the scaffolds, and SEM analysis was performed in the first 30 min, showing already large differences in cell attachment to the scaffold, yet no quantitative analysis was performed.

Owen et al. [109] and Kim et al. [110] both used an acrylic acid enriched O_2 low-pressure RF system to deposit acid rich coatings on their scaffolds with 800 and 450 µm pore diameters, respectively. Owen et al. used the scaffolds as such, seeding human mesenchymal progenitors. ALP activity was measured, showing that the stiffest samples combined with the acrylic acid resulted in the best osteogenic differentiation. Kim et al. introduced an extra step, immobilizing rhBMP-2 onto their scaffolds via simple incubation. MG-63 osteoblasts were seeded onto the

scaffold, and again ALP activity was measured. Results show an 80 % increase of activity compared to the coated scaffolds without immobilized protein and a 90 % increase compared with untreated scaffolds; results that are more pronounced compared to what was found by Owen et al.

Compared to the research performed on the plasma modification of traditionally fabricated scaffolds, the amount of papers on plasma-modified rapid-prototyped scaffolds is more recent and rather limited. The main reason for this is most likely the high acquisition cost of the rapid prototyping systems, ranging somewhere between 30.000 and 250.000 euro, combined with the fact that most of those systems can be found in biomaterials laboratories having limited experience with plasma technology. Those groups that did perform plasma grafting/polymerization on rapid-prototyped scaffolds have found very similar results compared to results found for modified traditional scaffolds. The main difference between the two fabrication techniques is that there is little to no mention of possible gradients in chemistry. This suggests that either: (1) it is not a problem thanks to the better pore interconnectivity or (2) no attention was paid to it. The latter seems more likely, based on the results found by Jacobs et al. [71] for a plasma-activated rapid-prototyped scaffolds. In either case, more structural studies are required to analyze this, as well as a transfer of some of the results found for traditional scaffolds to the rapid prototyped ones.

3.2.3. Electrospinning

Several of the experiments performed on other scaffolds have also been performed on electrospun scaffolds; therefore, this chapter part will mainly focus on those plasma grafting and polymerization strategies that have not been discussed for other scaffolds, while the rest will be summarized in Table 2 [141].

Author	scaffold material	reactor & gas	precursor	cell-type	effect
Shabani et al. [118]	PES	MW low pressure O_2	collagen grafting	somatic stem cells	better cell infiltration into the scaffold
Jeong et al. [119]	Silk	RF low pressure O_2	CH_4 polymerization	keratinocytes & fibroblasts	addition of O_2 into plasma increases initial adhesion
Park et al. [120]	PU	MW atmospheric plasma Ar	PLGA grafting	HUVEC	adhesion +100% proliferation +150% better endothelialization
Yao et al. [121]	PU	40 kHz low pressure plasma Ar	4-vinylpyridine + quaternization	S. aureus E. coli	Log5 and Log3 reduction in bacterial activity
Lopez-Pérez et al. [122]	PCL	RF low pressure O_2	vinyl phosphonic acid vinyl sulfonic acid	Saos-2 osteoblasts	higher vitronectin adsorption

Author	scaffold material	reactor & gas	precursor	cell-type	effect
					better proliferation for vinyl phosphonic acid
Chen et al. [123]	PLLA	DC low pressure pulsed system O_2	gelatin grafted	chondrocytes	4* increase in cell viability 4* increase in collagen production
Jia et al. [124]	PCL	RF low pressure Ar	soluble eggshell protein	human dermal fibroblasts	more elongated morphology
Park et al. [125]	PLA	RF low pressure O_2	acrylic acid polymerization	NIH 3T3	better adhesion and proliferation + complete coverage of the scaffold pores within 6 days
Paletta et al. [126]	PLLA	RF low pressure O_2	cyclic RGD grafted	mesenchymal stem cells	no enhanced differentiation due to limited RGD surface densities
Seyedjafari et al. [127]	PLLA	MW low pressure O_2	nanohydroxyapatite grafted	unrestricted somatic stem cells in-vivo	Higher ALP and mineralization + ossification and formation of trabeculi
Santos et al. [128]	Starch (100μm)	RF low pressure Ar	vitronectin & fibronectin adsorption	HUVEC	better proliferation higher concentration endothelial growth factors higher cadherin activity
Ma et al. [129]	PCL	RF low pressure air	gelatin grafted	endothelial cells	better morphology aligned along fibers
Ghaedi et al. [130]	PLA	MW low pressure O_2	collagen grafted	hepatocytes	efficient differentiation into osteoblasts and adipocytes
Ai et al. [131]	PHVB	MW low pressure O_2	collagen grafted	unrestricted somatic stem cells	enhanced proliferation

Table 2. Overview of literature on plasma polymerization/grafting on ES substrates not discussed within the text.

The flexibility of nanofibers allows them to be used in a wider variety of tissue engineering applications. An example that has been amply studied is the vascular graft. One aspect that is critical for such applications is the haemocompatibility. Wang et al. [111] and Cheng et al. [112] both used low-pressure RF systems to immobilize heparin on silk nanofibers and PLLA nanofibers, respectively. Whereas Wang et al. used an Ar plasma, Cheng et al. used an Ar/NH$_3$ + H$_2$ plasma. Both claim a high-grafting efficiency, however, the study of Cheng et al. shows that their treatment strategy results in a 10-times higher adsorption rate compared to

an Ar plasma treatment. In-vitro tests with blood platelets showed a significant decrease in platelet attachment compared to untreated scaffolds (Cheng et al.) and a four-time longer coagulation time (Wang et al.). Finally, the seeding of bovine aortic endothelial cells showed an enhanced cell infiltration into the scaffold independent of the level of heparin immobilized, suggesting that the addition of ammonia to the plasma is unnecessary (Cheng et al.). Wang et al. tested their scaffolds in-vivo, showing excellent biocompatibility with only minor signs of inflammation. He et al. [113] used a low-pressure ICP air plasma (30 W–5 min) to immobilize collagen rather than heparin onto their PCL-PLLA electrospun scaffolds (470 ± 130 nm) for vascular graft applications. Rhodamine-stained scaffolds show an excellent distribution of collagen throughout the scaffolds. Human coronary artery endothelial cells were seeded onto the scaffolds. The collagen-coated scaffolds stimulated the spreading, attachment, and overall viability combined with a good preservation of the phenotype.

Another application that has received considerable attention is the fabrication of e-spun scaffolds for nerve-guided regeneration. Delgado-Rivera et al. [114] used a plasma-patterning technique involving a PMDS mask to generate tracks of grafted lamilin on a polyamide nanofiber mesh. RG3.6 neural precursors were seeded onto both patterned and completely treated scaffolds. Fluorescence microscopy showed that the precursor cells almost exclusively stuck to the patterns growing in a bilateral fashion. Zandér et al. [115] used an air plasma treatment (18 W–5 min) on aligned PCL nanofibers with sub-μm dimensions followed by covalent immobilization of lamilin, using carbodiimide wet-chemistry. PC12 neuron-like differentiated cells were seeded onto the e-spun scaffolds. SEM imaging revealed significantly longer neurons on scaffolds that contain higher concentrations of lamilin. The authors do admit that they have no control on how the protein is folded onto the surface, thus having no indication on the efficiency of the immobilized lamilin, which could most likely be partially addressed via gene expression analysis.

Guex et al. [116] focused their research on the repair of damaged myocardium using PCL fibers coated with a CHO-type coating (ethylene + CO_2) rich with ester bonds (O/C = 0.35). Mesenchymal stem cells were seeded onto the scaffolds and then implanted into a rat model. Four weeks after implantation, scaffolds were analyzed, showing that the plasma-coated grafts significantly stabilized the cardiac function, resulted in attenuated dilation and gave cause to lower ejection fractioning and fractional shortening all compared to untreated scaffolds.

Nuhiji et al. [117] developed a multifunctional e-spun scaffold based on the immobilization of silane groups. Oxygen low pressure RF plasma pretreatment (63 Pa, 30 W, 3 min) resulted in a 10-fold increase in 3-aminopropyl-trimethoxysilane or 3-mercaptopropyl-trimethoxysilane. The silane-enriched surfaces were then immersed in a neutravidin-enriched buffer solution overnight, resulting in the effective immobilization of the neutravidin. CD-4 antibodies were seeded onto the scaffold, resulting in specific bonding. According to the authors, changing the neutravidin for other biomacromolecules such as proteins, enzymes, and antibodies should be easy, resulting in a multitool scaffold for biosensing and tissue engineering.

A wide variety of polymerizable precursors and biomacromolecules were selected by research groups around the world to be used for plasma polymerization and plasma grafting, respectively, on electrospun scaffolds. Compared to the plasma-activated scaffolds, results were far

more promising. In most cases, better cell proliferation rates were found on top of good initial cell adhesion and, when applicable, cell differentiation was drastically improved. In-vivo results were promising as well, with limited infection, good cell ingrowth, and, in a few cases, even good vascularization. Compared to other fabrication techniques, electrospinning has been studied quite extensively, resulting in a wider variety of possible treatment strategies.

4. Conclusion and outlook

A quite extensive overview has been given on the literature involving, in one way or the other, the use of non-thermal plasma technology for the modification of scaffolds for tissue engineering applications. In the past 15 years, research has evolved from "simple" plasma treatments, mostly improving cell adhesion, to quite elegant methods, often involving nothing more than a plasma step and some protein-enriched aqueous solutions. These methods resulted in scaffolds that were able to support cells that adhered better, proliferated better, and differentiated better compared to their untreated counterparts. These results show that plasma is a reliable and efficient tool that should be included in the toolbox of every tissue engineer. Comparing the three scaffold categories reviewed here, it is to our belief that the traditionally fabricated scaffolds will disappear over time, as the rapid prototyped scaffolds are superior in both pore interconnectivity and biomimetic design. Electrospinning is a technique that is complementary to the other two and should even be used in parallel with the others, combining both structural integrity and ECM-like support structures in one scaffold design. The challenges for the next few years will be to translate the technology from generic scaffolds to patient-customized scaffolds that could actually be used on patients. An increase in scaffold dimensions and complexity will be unavoidable and cause new and unexpected problems as well as increase problems still encountered today (such as gradient depositions). Secondly, more attention should be paid to the latest discoveries in cell biology, as this would accelerate the tuning process of what should be immobilized on a scaffolds' surface, thus addressing the issue that research conducted to this day is almost exclusively unidimensional, studying the effect of one component on cellular behavior, while it is known that most ques triggering the cellular metabolism are multicomponent systems. Gene expression analysis is becoming more popular, but should be systematically used to give researchers more fundamental insights into what their surface modification techniques are triggering on a molecular level. Thirdly, at this point, the level of in-vivo testing remains relatively low and should become more mainstream, as literature has shown that there are often large discrepancies between results found in-vitro and in-vivo.

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References

[1] Howard D, Buttery LD, Shakesheff KM, and Roberts SJ: Tissue engineering: strategies, stem cells and scaffolds. *Journal of Anatomy* 2008, 213(1):66–72.

[2] Blitterswijk CAV, Moroni L, Rouwkema J, Siddappa R, and Sohier J: In: Blitterswijk CV, Thomsen P, Lindahl A, Hubbell J, Williams DF, Cancedda R, Bruijn JDD, and Sohier J (Eds): *Tissue engineering*. Burlington: Academic Press, 2008, pp. xii–xxxvi.

[3] Novosel EC, Kleinhans C, and Kluger PJ: Vascularization is the key challenge in tissue engineering. *Advanced Drug Delivery Reviews* 2011, 63(4):300–311.

[4] Helsen JA, and Missirlis Y: Tissue engineering: Regenerative medicine. *Bio materials*, Springer, Verlag Berlin Heidelberg, 2010, pp. 269–289.

[5] Lanza R, Langer R, and Vacanti JP: *Principles of tissue engineering*. Academic Press, Burlington San Diego London, 2011.

[6] Mooney DJ, Baldwin DF, Suh NP, Vacanti JP, and Langer R: Novel approach to fabricate porous sponges of poly(d,l-lactic-co-glycolic acid) without the use of organic solvents. *Biomaterials*, 1996, 17(14):1417–1422.

[7] Brown RA: *Extreme tissue engineering: concepts and strategies for tissue fabrication*. Wiley, Oxford Chichester Hoboken, 2013.

[8] Hay ED: *Cell biology of extracellular matrix*. Springer, 2013.

[9] Hynes RO: The extracellular matrix: not just pretty fibrils. *Science (New York, N.Y.)*, 2009, 326(5957):1216–1219.

[10] O'Connor C, Adams JU, and Fairman J: *Essentials of cell biology*. Cambridge: NPG Education, 2010.

[11] Alberts B, Bray D, Hopkin K, Johnson A, Lewis J, Raff M, Roberts K, and Walter P: *Essential cell biology*. Garland Science, New York, 2013.

[12] Soderling TR, Chang B, and Brickey D: Cellular signaling through multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. *Journal of Biological Chemistry*, 2001, 276(6):3719–3722.

[13] Lutolf MP: Integration column: artificial ECM: expanding the cell biology toolbox in 3D. *Integrative Biology*, 2009, 1(3):235–241.

[14] Desmet T, Morent R, Geyter ND, Leys C, Schacht E, and Dubruel P: Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review. *Biomacromolecules*, 2009, 10(9):2351–2378.

[15] Morent R, De Geyter N, Desmet T, Dubruel P, and Leys C: Plasma surface modification of biodegradable polymers: a review. *Plasma Processes and Polymers*, 2011, 8(3): 171–190.

[16] Cools P, De Geyter N, and Morent R: PLA enhanced via plasma technology: a review. *New Developments in Polylactic Acid Research*, Nova Science, New York, 2015, pp. 79–110.

[17] Cools P, Morent R, and De Geyter N: Plasma modified textiles for biomedical applications. *Advances in Bioengineering,* InTechOpen, Rijeka, 2014, pp.117–148.

[18] Elloumi-Hannachi I, Yamato M, and Okano T: Cell sheet engineering: a unique nanotechnology for scaffold-free tissue reconstruction with clinical applications in regenerative medicine. *Journal of internal medicine*, 2010, 267(1):54–70.

[19] Drury JL, and Mooney DJ: Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*, 2003, 24(24):4337–4351.

[20] Billiet T, Vandenhaute M, Schelfhout J, Van Vlierberghe S, and Dubruel P: A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials*, 2012, 33(26):6020–6041.

[21] Lutolf M, and Hubbell J: Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotechnology*, 2005, 23(1):47–55.

[22] Hubbell JA: Biomaterials in tissue engineering. *Nature Biotechnology*, 1995, 13(6):565–576.

[23] Hodde J: Naturally occurring scaffolds for soft tissue repair and regeneration. *Tissue Engineering*, 2002, 8(2):295–308.

[24] Balakrishnan B, and Banerjee R: Biopolymer-based hydrogels for cartilage tissue engineering. *Chemical Reviews*, 2011, 111(8):4453–4474.

[25] Hollister SJ: Porous scaffold design for tissue engineering. *Nature Materials*, 2005, 4(7):518–524.

[26] Mironov V, Visconti RP, Kasyanov V, Forgacs G, Drake CJ, and Markwald RR: Organ printing: tissue spheroids as building blocks. *Biomaterials*, 2009, 30(12):2164–2174.

[27] Dhandayuthapani B, Yoshida Y, Maekawa T, and Kumar DS: Polymeric scaffolds in tissue engineering application: a review. *International Journal of Polymer Science*, 2011, 2011:1–19.

[28] Cheung H-Y, Lau K-T, Lu T-P, and Hui D: A critical review on polymer-based bio-engineered materials for scaffold development. *Composites Part B: Engineering*, 2007, 38(3):291–300.

[29] Tanner K: Bioactive composites for bone tissue engineering. Proceedings of the Institution of Mechanical Engineers. *Part H: Journal of Engineering in Medicine*, 2010, 224(12):1359–1372.

[30] Yeong W-Y, Chua C-K, Leong K-F, and Chandrasekaran M: Rapid prototyping in tissue engineering: challenges and potential. *Trends in Biotechnology*, 2004, 22(12):643–652.

[31] Claase MB, de Bruijn JD, Grijpma DW, and Feijen J: Ectopic bone formation in cell-seeded poly(ethylene oxide)/poly(butylene terephthalate) copolymer scaffolds of varying porosity. *Journal of Materials Science: Materials in Medicine*, 2007, 18(7):1299–1307.

[32] Quirk RA, France RM, Shakesheff KM, and Howdle SM: Supercritical fluid technologies and tissue engineering scaffolds. *Current Opinion in Solid State and Materials Science*, 2004, 8(3–4):313–321.

[33] Tai H, Mather ML, Howard D, Wang W, White LJ, Crowe JA, Morgan SP, Chandra A, Williams DJ, and Howdle SM: Control of pore size and structure of tissue engineering scaffolds produced by supercritical fluid processing. *European Cells & Materials*, 2007, 14:64–77.

[34] Bártolo PJ, Domingos M, Patrício T, Cometa S, and Mironov V: Biofabrication Strategies for Tissue Engineering. *Advances on Modeling in Tissue Engineering*, Springer, Dordrecht Heidelberg London New York, 2011, pp. 137–176.

[35] Abdelaal OA, and Darwish SM: *Characterization and Development of Biosystems and Biomaterials*. Springer, 2013, pp. 33–54.

[36] Declercq HA, Gorski TL, Tielens SP, Schacht EH, and Cornelissen MJ: Encapsulation of osteoblast seeded microcarriers into injectable, photopolymerizable three-dimensional scaffolds based on d,l-lactide and ε-caprolactone. *Biomacromolecules*, 2005, 6(3): 1608–1614.

[37] Lippens E, Swennen I, Gironès J, Declercq H, Vertenten G, Vlaminck L, Gasthuys F, Schacht E, and Cornelissen R: Cell survival and proliferation after encapsulation in a chemically modified Pluronic® F127 hydrogel. *Journal of Biomaterials Applications*, 2013, 27(7):828–839.

[38] Li D, and Xia Y: Electrospinning of nanofibers: reinventing the wheel? *Advanced Materials*, 2004, 16(14):1151–1170.

[39] Pham QP, Sharma U, and Mikos AG: Electrospinning of polymeric nanofibers for tissue engineering applications: a review. *Tissue Engineering*, 2006, 12(5):1197–1211.

[40] Cipitria A, Skelton A, Dargaville T, Dalton P, and Hutmacher D: Design, fabrication and characterization of PCL electrospun scaffolds—a review. *Journal of Materials Chemistry*, 2011, 21(26):9419–9453.

[41] Agarwal S, Wendorff JH, and Greiner A: Use of electrospinning technique for biomedical applications. *Polymer*, 2008, 49(26):5603–5621.

[42] Martins A, Reis R, and Neves N: Electrospinning: processing technique for tissue engineering scaffolding. *International Materials Reviews*, 2008, 53(5):257–274.

[43] Ma Z, Kotaki M, Inai R, and Ramakrishna S: Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Engineering*, 2005, 11(1–2):101–109.

[44] Jacobs T, Morent R, De Geyter N, Dubruel P, and Leys C: Plasma surface modification of biomedical polymers: influence on cell-material interaction. *Plasma Chemistry and Plasma Processing*, 2012, 32(5):1039–1073.

[45] Cheruthazhekatt S, Černák M, Slavíček P, and Havel J: Gas plasmas and plasma modified materials in medicine. *Journal of Applied Biomedicine*, 2010, 8(2):55–66.

[46] Chu PK, Chen J, Wang L, and Huang N: Plasma-surface modification of biomaterials. *Materials Science and Engineering: R: Reports*, 2002, 36(5):143–206.

[47] Siow KS, Britcher L, Kumar S, Griesser HJ, Plasma methods for the generation of chemically reactive surfaces for biomolecule immobilization and cell colonization—a review. *Plasma Processes and Polymers*, 2006, 3(6-7):392–418.

[48] Noeske M, Degenhardt J, Strudthoff S, and Lommatzsch U: Plasma jet treatment of five polymers at atmospheric pressure: surface modifications and the relevance for adhesion. *International Journal of Adhesion and Adhesives*, 2004, 24(2):171–177.

[49] Hegemann D, Brunner H, and Oehr C: Plasma treatment of polymers for surface and adhesion improvement. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, 2003, 208:281–286.

[50] Chim H, Ong JL, Schantz JT, Hutmacher DW, and Agrawal C: Efficacy of glow discharge gas plasma treatment as a surface modification process for three-dimensional poly(d,l-lactide) scaffolds. *Journal of Biomedical Materials Research Part A*, 2003, 65(3): 327–335.

[51] Köse GT, Kenar H, Hasırcı N, and Hasırcı V: Macroporous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) matrices for bone tissue engineering. *Biomaterials*, 2003, 24(11): 1949–1958.

[52] Claase MB, Grijpma DW, Mendes SC, de Bruijn JD, and Feijen J: Porous PEOT/PBT scaffolds for bone tissue engineering: preparation, characterization, and in vitro bone

marrow cell culturing. *Journal of Biomedical Materials Research Part A*, 2003, 64(2):291–300.

[53]   Yang J, Shi G, Bei J, Wang S, Cao Y, Shang Q, Yang G, and Wang W: Fabrication and surface modification of macroporous poly(l-lactic acid) and poly(l-lactic-co-glycolic acid) (70/30) cell scaffolds for human skin fibroblast cell culture. *Journal of Biomedical Materials Research*, 2002, 62(3):438–446.

[54]   Choi Y-R, Kwon J-S, Song D-H, Choi EH, Lee Y-K, Kim K-N, and Kim K-M: Surface modification of biphasic calcium phosphate scaffolds by non-thermal atmospheric pressure nitrogen and air plasma treatment for improving osteoblast attachment and proliferation. *Thin Solid Films*, 2013, 547:235–240.

[55]   Ring A, Langer S, Schaffran A, Stricker I, Awakowicz P, Steinau H-U, and Hauser J: Enhanced neovascularization of dermis substitutes via low-pressure plasma-mediated surface activation. *Burns*, 2010, 36(8):1222–1227.

[56]   Shah AR, Cornejo A, Guda T, Sahar DE, Stephenson SM, Chang S, Krishnegowda NK, Sharma R, and Wang HT: Differentiated adipose-derived stem cell cocultures for bone regeneration in polymer scaffolds in vivo. *Journal of Craniofacial Surgery*, 2014, 25(4):1504–1509.

[57]   Safinia L, Datan N, Höhse M, Mantalaris A, and Bismarck A: Towards a methodology for the effective surface modification of porous polymer scaffolds. *Biomaterials*, 2005, 26(36):7537–7547.

[58]   Wan Y, Tu C, Yang J, Bei J, and Wang S: Influences of ammonia plasma treatment on modifying depth and degradation of poly(l-lactide) scaffolds. *Biomaterials*, 2006, 27(13):2699–2704.

[59]   Safinia L, Wilson K, Mantalaris A, and Bismarck A: Atmospheric plasma treatment of porous polymer constructs for tissue engineering applications. *Macromolecular Bioscience*, 2007, 7(3):315–327.

[60]   Han I, Vagaska B, Lee DH, Park BJ, Shin S-W, Kim JK, Lee SJ, and Park J-C: Pre-wetting process with helium atmospheric pressure glow discharge for three-dimensional porous scaffold. *Macromolecular Research*, 2011, 19(7):711–715.

[61]   Bak T-Y, Kook M-S, Jung S-C, and Kim B-H: Biological effect of gas plasma treatment on CO_2 gas foaming/salt leaching fabricated porous polycaprolactone scaffolds in bone tissue engineering. *Journal of Nanomaterials*, 2014, 2014:1–6.

[62]   Sardella E, Gristina R, Senesi GS, d'Agostino R, and Favia P: Homogeneous and micro-patterned plasma-deposited PEO-like coatings for biomedical surfaces. *Plasma Processes and Polymers*, 2004, 1(1):63–72.

[63]   Trizio I, Intranuovo F, Gristina R, Dilecce G, and Favia P: He/O_2 atmospheric pressure plasma jet treatments of pcl scaffolds for tissue engineering and regenerative

medicine. *Plasma Processes and Polymers*, 2015, Early View Publication; these are NOT the final page numbers, use DOI for citation DOI: 10.1002/ppap.201500104.

[64] Yang S, Leong K-F, Du Z, and Chua C-K: The design of scaffolds for use in tissue engineering. Part II. Rapid prototyping techniques. *Tissue Engineering*, 2002, 8(1):1–11.

[65] Landers R, Hübner U, Schmelzeisen R, and Mülhaupt R: Rapid prototyping of scaffolds derived from thermoreversible hydrogels and tailored for applications in tissue engineering. *Biomaterials*, 2002, 23(23):4437–4447.

[66] Hutmacher DW: Scaffolds in tissue engineering bone and cartilage. *Biomaterials*, 2000, 21(24):2529–2543.

[67] Wagner M, Kiapur N, Wiedmann-Al-Ahmad M, Hübner U, Al-Ahmad A, Schön R, Schmelzeisen R, Mülhaupt R, and Gellrich NC: Comparative in vitro study of the cell proliferation of ovine and human osteoblast-like cells on conventionally and rapid prototyping produced scaffolds tailored for application as potential bone replacement material. *Journal of Biomedical Materials Research Part A*, 2007, 83A(4):1154–1164.

[68] Moroni L, Curti M, Welti M, Korom S, Weder W, De Wijn JR, and Van Blitterswijk CA: Anatomical 3D fiber-deposited scaffolds for tissue engineering: designing a neotrachea. *Tissue Engineering*, 2007, 13(10):2483–2493.

[69] Yildirim ED, Besunder R, Guceri S, Allen F, and Sun W: Fabrication and plasma treatment of 3D polycaprolactane tissue scaffolds for enhanced cellular function. *Virtual and Physical Prototyping*, 2008, 3(4):199–207.

[70] Yildirim ED, Pappas D, Güçeri S, and Sun W: Enhanced cellular functions on polycaprolactone tissue scaffolds by O_2 plasma surface modification. *Plasma Processes and Polymers*, 2011, 8(3):256–267.

[71] Jacobs T, Declercq H, De Geyter N, Cornelissen R, Dubruel P, Leys C, and Morent R: Improved cell adhesion to flat and porous plasma-treated poly-ε-caprolactone samples. *Surface and Coatings Technology*, 2013, 232:447–455.

[72] Jacobs T, Morent R, De Geyter N, Desmet T, Dubruel P, and Leys C: Visualization of the penetration depth of plasma in three-dimensional porous PCL scaffolds. *IEEE Transactions on Plasma Science*, 2011, 39(11(1)):2792–2793.

[73] Baker SC, Atkin N, Gunning PA, Granville N, Wilson K, Wilson D, and Southgate J: Characterisation of electrospun polystyrene scaffolds for three-dimensional in vitro biological studies. *Biomaterials*, 2006, 27(16):3136–3146.

[74] France R, and Short R: Plasma treatment of polymers effects of energy transfer from an argon plasma on the surface chemistry of poly(styrene), low density poly(ethylene), poly(propylene) and poly(ethylene terephthalate). *Journal of the Chemical Society, Faraday Transactions*, 1997, 93(17):3173–3178.

[75] Park H, Lee KY, Lee SJ, Park KE, and Park WH: Plasma-treated poly(lactic-co-glycolic acid) nanofibers for tissue engineering. *Macromolecular Research*, 2007, 15(3):238–243.

[76] Yang F, Wolke JGC, and Jansen JA: Biomimetic calcium phosphate coating on electrospun poly(ε-caprolactone) scaffolds for bone tissue engineering. *Chemical Engineering Journal*, 2008, 137(1):154–161.

[77] Prabhakaran MP, Venugopal J, Chan CK, and Ramakrishna S: Surface modified electrospun nanofibrous scaffolds for nerve tissue engineering. *Nanotechnology*, 2008, 19(45):455102.

[78] Park H, Lee JW, Park KE, Park WH, and Lee KY: Stress response of fibroblasts adherent to the surface of plasma-treated poly(lactic-co-glycolic acid) nanofiber matrices. *Colloids and Surfaces B: Biointerfaces*, 2010, 77(1):90–95.

[79] de Valence S, Tille J-C, Chaabane C, Gurny R, Bochaton-Piallat M-L, Walpoth BH, and Möller M: Plasma treatment for improving cell biocompatibility of a biodegradable polymer scaffold for vascular graft applications. *European Journal of Pharmaceutics and Biopharmaceutics*, 2013, 85(1):78–86.

[80] Yan D, Jones J, Yuan X, Xu X, Sheng J, Lee JM, Ma G, and Yu Q: Plasma treatment of electrospun PCL random nanofiber meshes (NFMs) for biological property improvement. *Journal of Biomedical Materials Research Part A*, 2013, 101(4):963–972.

[81] Dolci LS, Quiroga SD, Gherardi M, Laurita R, Liguori A, Sanibondi P, Fiorani A, Calzà L, Colombo V, and Focarete ML: Carboxyl surface functionalization of poly(L-lactic acid) electrospun nanofibers through atmospheric non-thermal plasma affects fibroblast morphology. *Plasma Processes and Polymers*, 2014, 11(3):203–213.

[82] Sankar D, Shalumon K, Chennazhi K, Menon D, and Jayakumar R: Surface plasma treatment of poly(caprolactone) micro, nano, and multiscale fibrous scaffolds for enhanced osteoconductivity. *Tissue Engineering Part A*, 2014, 20(11–12):1689–1702.

[83] Jeon HJ, Lee H, and Kim GH: Nano-sized surface patterns on electrospun microfibers fabricated using a modified plasma process for enhancing initial cellular activities. *Plasma Processes and Polymers*, 2014, 11(2):142–148.

[84] Venugopal J, Low S, Choon AT, Kumar AB, and Ramakrishna S: Electrospun-modified nanofibrous scaffolds for the mineralization of osteoblast cells. *Journal of Biomedical Materials Research Part A*, 2008, 85(2):408–417.

[85] Cheng Q, Lee BL-P, Komvopoulos K, Yan Z, and Li S: Plasma surface chemical treatment of electrospun poly(L-lactide) microfibrous scaffolds for enhanced cell adhesion, growth, and infiltration. *Tissue Engineering Part A*, 2013, 19(9–10):1188–1198.

[86] Yildirim ED, Ayan H, Vasilets VN, Fridman A, Guceri S, and Sun W: Effect of dielectric barrier discharge plasma on the attachment and proliferation of osteoblasts cul-

tured over poly(ε-caprolactone) scaffolds. *Plasma Processes and Polymers*, 2008, 5(1): 58–66.

[87] Santerre JP, Woodhouse K, Laroche G, and Labow RS: Understanding the biodegradation of polyurethanes: from classical implants to tissue engineering materials. *Biomaterials*, 2005, 26(35):7457–7470.

[88] Zandén C, Voinova M, Gold J, Mörsdorf D, Bernhardt I, and Liu J: Surface characterisation of oxygen plasma treated electrospun polyurethane fibres and their interaction with red blood cells. *European Polymer Journal*, 2012, 48(3):472–482.

[89] Zandén C, Erkenstam NH, Padel T, Wittgenstein J, Liu J, and Kuhn HG: Stem cell responses to plasma surface modified electrospun polyurethane scaffolds. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2014, 10(5):949–958.

[90] Ardeshirylajimi A, Dinarvand P, Seyedjafari E, Langroudi L, Adegani FJ, and Soleimani M: Enhanced reconstruction of rat calvarial defects achieved by plasma-treated electrospun scaffolds and induced pluripotent stem cells. *Cell and Tissue Research*, 2013, 354(3):849–860.

[91] Baek HS, Park YH, Ki CS, Park J-C, and Rah DK: Enhanced chondrogenic responses of articular chondrocytes onto porous silk fibroin scaffolds treated with microwave-induced argon plasma. *Surface and Coatings Technology*, 2008, 202(22):5794–5797.

[92] Cheon YW, Lee WJ, Baek HS, Lee YD, Park JC, Park YH, Ki CS, Chung KH, and Rah DK: Enhanced chondrogenic responses of human articular chondrocytes onto silk fibroin/wool keratose scaffolds treated with microwave-induced argon plasma. *Artificial Organs*, 2010, 34(5):384–392.

[93] Barry JJA, Silva MMCG, Shakesheff KM, Howdle SM, and Alexander MR: Using plasma deposits to promote cell population of the porous interior of three-dimensional poly(d,l-lactic acid) tissue-engineering scaffolds. *Advanced Functional Materials*, 2005, 15(7):1134–1140.

[94] Intranuovo F, Howard D, White LJ, Johal RK, Ghaemmaghami AM, Favia P, Howdle SM, Shakesheff KM, Alexander MR, Uniform cell colonization of porous 3-D scaffolds achieved using radial control of surface chemistry. *Acta Biomaterialia*, 2011, 7(9): 3336–3344.

[95] Intranuovo F, Gristina R, Brun F, Mohammadi S, Ceccone G, Sardella E, Rossi F, Tromba G, and Favia P: Plasma modification of PCL porous scaffolds fabricated by solvent-casting/particulate-leaching for tissue engineering. *Plasma Processes and Polymers*, 2014, 11(2):184–195.

[96] Ju YM, Park K, Son JS, Kim JJ, Rhie JW, and Han DK: Beneficial effect of hydrophilized porous polymer scaffolds in tissue-engineered cartilage formation. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 2008, 85(1):252–260.

[97] Park K, Jung HJ, Kim J-J, and Han DK: Effect of surface-activated PLLA scaffold on apatite formation in simulated body fluid. *Journal of Bioactive and Compatible Polymers*, 2010, 25(1):27–39.

[98] Demirbilek ME, Demirbilek M, Karahaliloğlu Z, Erdal E, Vural T, Yalçın E, Sağlam N, and Denkbaş EB: Oxidative stress parameters of L929 cells cultured on plasma-modified PDLLA scaffolds. *Applied Biochemistry and Biotechnology*, 2011, 164(6):780–792.

[99] Shen H, Hu X, Yang F, Bei J, and Wang S: The bioactivity of rhBMP-2 immobilized poly(lactide-co-glycolide) scaffolds. *Biomaterials*, 2009, 30(18):3150–3157.

[100] Woo YI, Lee MH, Kim H-L, Park J-C, Han D-W, Kim JK, Tsubaki K, Chung K-H, Hyun SO, and Yang Y-I: Cellular responses and behaviors of adipose-derived stem cells onto β-glucan and PLGA composites surface-modified by microwave-induced argon plasma. *Macromolecular Research*, 2010, 18(1):90–93.

[101] Hu Y, Winn SR, Krajbich I, and Hollinger JO: Porous polymer scaffolds surface-modified with arginine-glycine-aspartic acid enhance bone cell attachment and differentiation in vitro. *Journal of Biomedical Materials Research Part A*, 2003, 64(3):583–590.

[102] Zheng L, Yang F, Shen H, Hu X, Mochizuki C, Sato M, Wang S, and Zhang Y: The effect of composition of calcium phosphate composite scaffolds on the formation of tooth tissue from human dental pulp stem cells. *Biomaterials*, 2011, 32(29):7053–7059.

[103] Berneel E, Desmet T, Declercq H, Dubruel P, and Cornelissen M: Double protein-coated poly-ε-caprolactone scaffolds: successful 2D to 3D transfer. *Journal of Biomedical Materials Research Part A*, 2012, 100(7):1783–1791.

[104] Declercq HA, Desmet T, Dubruel P, and Cornelissen MJ: The role of scaffold architecture and composition on the bone formation by adipose-derived stem cells. *Tissue Engineering Part A*, 2013, 20(1–2):434–444.

[105] Declercq HA, Desmet T, Berneel EE, Dubruel P, and Cornelissen MJ: Synergistic effect of surface modification and scaffold design of bioplotted 3-D poly-ε-caprolactone scaffolds in osteogenic tissue engineering. *Acta Biomaterialia*, 2013, 9(8):7699–7708.

[106] Van Bael S, Desmet T, Chai YC, Pyka G, Dubruel P, Kruth J-P, and Schrooten J: In vitro cell-biological performance and structural characterization of selective laser sintered and plasma surface functionalized polycaprolactone scaffolds for bone regeneration. *Materials Science and Engineering: C*, 2013, 33(6):3404–3412.

[107] Sousa I, Mendes A, Pereira RF, and Bártolo PJ: Collagen surface modified poly(ε-caprolactone) scaffolds with improved hydrophilicity and cell adhesion properties. *Materials Letters*, 2014, 134:263–267.

[108] Nebe B, Cornelsen M, Quade A, Weissmann V, Kunz F, Ofe S, Schroeder K, Finke B, Seitz H, and Bergemann C: Osteoblast Behavior In Vitro in Porous Calcium Phos-

phate Composite Scaffolds, Surface Activated with a Cell Adhesive Plasma Polymer Layer. Materials science forum. Trans Tech Publications, 2012, pp. 566–571.

[109] Owen R, Sherborne C, Paterson T, Green NH, Reilly GC, and Claeyssens F: Emulsion templated scaffolds with tunable mechanical properties for bone tissue engineering. *Journal of the Mechanical Behavior of Biomedical Materials*, 2015, 54:159–172.

[110] Kim BH, Myung SW, Jung SC, and Ko YM: Plasma surface modification for immobilization of bone morphogenic protein-2 on polycaprolactone scaffolds. *Japanese Journal of Applied Physics*, 2013, 52(11S):11NF01.

[111] Wang S, Zhang Y, Wang H, and Dong Z: Preparation, characterization and biocompatibility of electrospinning heparin-modified silk fibroin nanofibers. *International Journal of Biological Macromolecules*, 2011, 48(2):345–353

[112] Cheng Q, Komvopoulos K, and Li S: Plasma-assisted heparin conjugation on electrospun poly(l-lactide) fibrous scaffolds. *Journal of Biomedical Materials Research Part A*, 2014, 102(5):1408–1414.

[113] He W, Ma Z, Yong T, Teo WE, and Ramakrishna S: Fabrication of collagen-coated biodegradable polymer nanofiber mesh and its potential for endothelial cells growth. *Biomaterials*, 2005, 26(36):7606–7615.

[114] Delgado-Rivera R, Griffin J, Ricupero CL, Grumet M, Meiners S, and Uhrich KE: Microscale plasma-initiated patterning of electrospun polymer scaffolds. *Colloids and Surfaces B: Biointerfaces*, 2011, 84(2):591–596.

[115] Zander NE, Orlicki JA, Rawlett AM, Beebe Jr. TP, Quantification of protein incorporated into electrospun polycaprolactone tissue engineering scaffolds. *ACS Applied Materials & Interfaces*, 2012, 4(4):2074–2081.

[116] Guex A, Frobert A, Valentin J, Fortunato G, Hegemann D, Cook S, Carrel T, Tevaearai H, and Giraud M-N: Plasma-functionalized electrospun matrix for biograft development and cardiac function stabilization. *Acta Biomaterialia*, 2014, 10(7):2996–3006.

[117] Nuhiji E, Wong CS, Sutti A, Lin T, Kirkland M, and Wang X: Biofunctionalization of 3D nylon 6, 6 scaffolds using a two-step surface modification. *ACS Applied Materials & Interfaces*, 2012, 4(6):2912–2919.

[118] Shabani I, Haddadi-Asl V, Seyedjafari E, Babaeijandaghi F, and Soleimani M: Improved infiltration of stem cells on electrospun nanofibers. *Biochemical and Biophysical Research Communications*, 2009, 382(1):129–133.

[119] Jeong L, Yeo I-S, Kim HN, Yoon YI, Jang DH, Jung SY, Min B-M, and Park WH: Plasma-treated silk fibroin nanofibers for skin regeneration. *International Journal of Biological Macromolecules*, 2009, 44(3):222–228.

[120] Park BJ, Seo HJ, Kim J, Kim H-L, Kim JK, Choi JB, Han I, Hyun SO, Chung K-H, and Park J-C: Cellular responses of vascular endothelial cells on surface modified polyur-

ethane films grafted electrospun PLGA fiber with microwave-induced plasma at atmospheric pressure. *Surface and Coatings Technology*, 2010, 205:S222–S226.

[121] Yao C, Li X, Neoh K, Shi Z, and Kang E: Surface modification and antibacterial activity of electrospun polyurethane fibrous membranes with quaternary ammonium moieties. *Journal of Membrane Science*, 2008, 320(1):259–267.

[122] López-Pérez PM, Da Silva RM, Sousa RA, Pashkuleva I, and Reis RL: Plasma-induced polymerization as a tool for surface functionalization of polymer scaffolds for bone tissue engineering: an in vitro study. *Acta Biomaterialia*, 2010, 6(9):3704–3712.

[123] Chen J-P, and Su C-H: Surface modification of electrospun PLLA nanofibers by plasma treatment and cationized gelatin immobilization for cartilage tissue engineering. *Acta Biomaterialia*, 2011, 7(1):234–243.

[124] Jia J, Duan YY, Yu J, and Lu JW: Preparation and immobilization of soluble eggshell membrane protein on the electrospun nanofibers to enhance cell adhesion and growth. *Journal of Biomedical Materials Research Part A*, 2008, 86(2):364–373.

[125] Park K, Jung HJ, Kim J-J, Ahn K-D, Han DK, and Ju YM: Acrylic acid-grafted hydrophilic electrospun nanofibrous poly(l-lactic acid) scaffold. *Macromolecular Research*, 2006, 14(5):552–558.

[126] Paletta JRJ, Bockelmann S, Walz A, Theisen C, Wendorff JH, Greiner A, Fuchs-Winkelmann S, and Schofer MD: RGD-functionalisation of PLLA nanofibers by surface coupling using plasma treatment: influence on stem cell differentiation. *Journal of Materials Science: Materials in Medicine*, 2010, 21(4):1363–1369.

[127] Seyedjafari E, Soleimani M, Ghaemi N, and Shabani I: Nanohydroxyapatite-coated electrospun poly(l-lactide) nanofibers enhance osteogenic differentiation of stem cells and induce ectopic bone formation. *Biomacromolecules*, 2010, 11(11):3118–3125.

[128] Santos M, Pashkuleva I, Alves C, Gomes ME, Fuchs S, Unger RE, Reis R, and Kirkpatrick CJ: Surface-modified 3D starch-based scaffold for improved endothelialization for bone tissue engineering. *Journal of Materials Chemistry*, 2009, 19(24):4091–4101.

[129] Ma Z, He W, Yong T, and Ramakrishna S: Grafting of gelatin on electrospun poly(caprolactone) nanofibers to improve endothelial cell spreading and proliferation and to control cell orientation. *Tissue Engineering*, 2005, 11(7–8):1149–1158.

[130] Ghaedi M, Soleimani M, Shabani I, Duan Y, and Lotfi A: Hepatic differentiation from human mesenchymal stem cells on a novel nanofiber scaffold. *Cellular and Molecular Biology Letters*, 2012, 17(1):89–106.

[131] Ai J, Heidari KS, Ghorbani F, Ejazi F, Biazar E, Asefnejad A, Pourshamsian K, and Montazeri M: Fabrication of coated-collagen electrospun PHBV nanofiber film by plasma method and its cellular study. *Journal of Nanomaterials*, 2011, 2011:1–8.

[132] Reprinted from an Illustration from Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013 under the Creative Commons Attribution 3.0 Unported license.

[133] Reprinted from Soft Matter, 2007, Girard PP, Cavalcanti-Adam EA, Kemkemer R, and Spatz JP: Cellular chemomechanics at interfaces: sensing, integration and response, Copyright © 2007, with permission from Royal Society of Chemistry.

[134] Reprinted from Nature Nanotechnology, 2010, Dvir T, Timko BP, Kohane DS, and Langer R: Nanotechnological strategies for engineering complex tissues, Copyright © 2010, with permission from Nature Publishing Group.

[135] Reprinted from Chemical Society Reviews, 2009, Place ES, George JH, Williams CK, and Stevens MM: Synthetic polymer scaffolds for tissue engineering. Copyright © 2009, with permission from Royal Society of Chemistry.

[136] Reprinted from Plasma Processes and Polymers, 2014, Intranuovo F, Gristina R, Brun F, Mohammadi S, Ceccone G, Sardella E, Rossi F, Tromba G, and Favia P: Plasma modification of PCL porous scaffolds fabricated by solvent-casting/particulate-leaching for tissue engineering. Copyright © 2014, with permission from Wiley.

[137] Reprinted from Surface and Coatings Technology, 2013, Jacobs T, Declercq H, De Geyter N, Cornelissen R, Dubruel P, Leys C, and and Morent R: Improved cell adhesion to flat and porous plasma-treated poly-ε-caprolactone samples. Copyright © 2013, with permission from Elsevier.

[138] Reprinted from Plasma Processes and Polymers, 2007, Yildirim ED, Ayan H, Vasilets VN, Fridman A, Guceri S, and Sun W: Effect of dielectric barrier discharge plasma on the attachment and proliferation of osteoblasts cultured over poly(ε-caprolactone) scaffolds. Copyright © 2007, with permission from Wiley.

[139] Reprinted from Plasma Processes and Polymers, 2013, Jeon HJ, Lee H, and Kim GH: Nano-sized surface patterns on electrospun microfibers fabricated using a modified plasma process for enhancing initial cellular activities. Copyright © 2013, with permission from Wiley.

[140] Reprinted from Advanced Functional Materials, 2005, Barry JJA, Silva MMCG, Shakesheff KM, Howdle SM, and Alexander MR: Using plasma deposits to promote cell population of the porous interior of three-dimensional poly(d,l-lactic acid) tissue-engineering scaffolds. Copyright © 2005, with permission from Wiley.

[141] Reprinted from Journal of Biomedical Materials Research, 2012, Berneel E, Desmet T, Declercq H, Dubruel P, and Cornelissen M: Double protein-coated poly-ε-caprolactone scaffolds: successful 2D to 3D transfer. Copyright © 2012, with permission from Wiley.