

Review

## The Use of Natural Polymers in Tissue Engineering: A Focus on Electrospun Extracellular Matrix Analogues

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**Abstract:** Natural polymers such as collagens, elastin, and fibrinogen make up much of the body's native extracellular matrix (ECM). This ECM provides structure and mechanical integrity to tissues, as well as communicating with the cellular components it supports to help facilitate and regulate daily cellular processes and wound healing. An ideal tissue engineering scaffold would not only replicate the structure of this ECM, but would also replicate the many functions that the ECM performs. In the past decade, the process of electrospinning has proven effective in creating non-woven ECM analogue scaffolds of micro to nanoscale diameter fibers from an array of synthetic and natural polymers. The ability of this fabrication technique to utilize the aforementioned natural polymers to create tissue engineering scaffolds has yielded promising results, both *in vitro* and *in vivo*, due in part to the enhanced bioactivity afforded by materials normally found within the human body. This review will present the process of electrospinning and describe the use of natural polymers in the creation of bioactive ECM analogues in tissue engineering.

**Keywords:** electrospinning; tissue engineering; natural polymers; collagen; elastin; fibrinogen; silk fibroin; extracellular matrix

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## 1. Introduction

While the process of electrospinning has been used commercially since the early 20<sup>th</sup> century, originally devised as a textile fabrication process, the rather recent ability to electrospin naturally occurring polymers has helped to reinvigorate the process and expand its potential applications. Electrospinning is lauded for its ability to economically and efficiently create non-woven meshes of sub-micron diameter fibers, particularly in the field of tissue engineering, where these fibrous scaffolds can replicate both the form and function of native extracellular matrix (ECM). While a synthetic polymer scaffold can mimic the structure of the native ECM, the ability to electrospin a naturally occurring protein such as collagen can provide cells with a recognizable, physiologically relevant platform from which to proliferate and ultimately remodel. With collagen constituting the majority of the human body's structural ECM, electrospun collagen has been used in a number of tissue engineering applications; everything from cardiovascular tissues, musculoskeletal tissues, skin, and nerve have used electrospun collagen fibers as the basis of their ECM analogues. Other natural polymers such as fibrinogen, often considered to be nature's provisional matrix due to its importance in the early stages of hemostasis and wound repair, have been electrospun and used for their excellent bioactive properties. This review will present the process of electrospinning, and describe the use of the natural polymers collagen, gelatin, elastin, fibrinogen, and silk fibroin (SF) in the creation of bioactive ECM analogues in tissue engineering.

### 1.1. The Native Extracellular Matrix

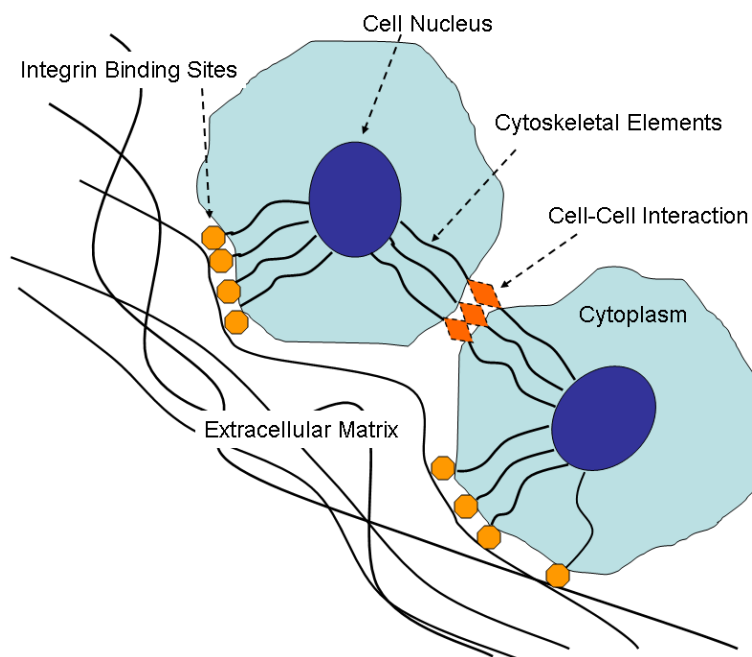
The tissues of the human body are composed of two main constituents: cells, which regulate body processes and perform normal maintenance and wound healing, and the ECM, a collection of macromolecules (Table 1) which serves as the major structural component of the body. This ECM makes up a large majority of tissue volume, and also provides much of a tissue's unique geometric shape, consisting primarily of fibers with diameters between 50 and 500 nm [1]. Composed of a dynamic, three-dimensional arrangement of polysaccharides and natural polymers (collagen, elastin, fibrinogen, *etc.*), ECM components are constantly being synthesized, secreted, oriented, and modified by the cellular components that they support. Historically, the function of native ECM was only believed to be as a structural framework for tissues. However it is now understood that the ECM, through interactions with receptors on the surfaces of cells, plays a large part in both day-to-day cellular activity and in wound healing. During development, cell-ECM interaction is responsible for pattern formation, morphogenesis, and phenotype acquisition and maintenance. During clot formation, wound healing, inflammation, formation of granulation tissue, and remodeling are all mediated by cell-ECM interaction. Cell adhesion, migration, growth, differentiation, and apoptosis are all controlled, in part, by the transmission of signals between the cell nucleus and the ECM. Additionally, growth factors and signaling molecules can be stored within the ECM, protecting them from premature degradation, or they can attach to the surface of the ECM to present themselves more efficiently to cell receptors [1-4].

**Table 1.** Major components of the native ECM, their locations, and functions in the body. Adapted from [3].

| Component                  | Location  | Function   |
|----------------------------|---|--|
| Collagen                   | Widely distributed                                | Key component of tissue architecture, provide tensile strength, cell-matrix interaction, matrix-matrix interaction       |
| Elastin                    | Highly elastic tissues (lung, blood vessel, skin) | Key component of tissue architecture, provide elasticity   |
| Proteoglycans              | Widely distributed                                | Cell-matrix interaction, matrix-matrix interaction, cell proliferation, cell migration                                   |
| Hyaluronan                 | Widely distributed                                | Cell-matrix interaction, matrix-matrix interaction, cell proliferation, cell migration                                   |
| Laminin                    | Basement membranes                                | Basement membrane component, cell migration  |
| Fibronectin                | Widely distributed                                | Component of tissue architecture, cell-matrix interaction, matrix-matrix interaction, cell proliferation, cell migration |
| Fibrinogen                 | Blood, sites of wound healing                     | Cell proliferation, cell migration, hemostasis   |
| Various Adhesion Molecules | Widely distributed                                | Mediate cell adhesion to matrix, mediate transmembrane signals   |

The multi-faceted cell-ECM communication takes place through both integrin and non-integrin membrane-bound receptors. This communication is complex and dynamic, and plays critical roles during development, wound healing, and environmental maintenance (Figure 1). Initial attraction and adhesion of cells to the ECM is induced by multiple, low affinity charge and hydrophobic interactions. Once cells have begun attaching, the spreading phase of adhesion is induced by heterodimeric transmembrane proteins, known as integrins, on the cell surface which bind to specific small peptide fragment sequences on the ECM. This connection allows cells to bind to the ECM, through focal adhesions, and promote direct communication between the two. Integrin binding is both specific and reversible and allows cells to differentiate, secrete and absorb matrix, and transmit signals [4]. The ECM has the ability to communicate with cells through “outside-in” signaling by sending signals across the cell membrane to soluble molecules in the cytoplasm and through direct connections with the cytoskeleton and into the cell nucleus. This direct contact allows for stronger, more specific signaling than through the release of diffusible signaling molecules. “Inside-out” cell-ECM interactions are also possible, and occur when changes within the cell result in a feedback which alters the activity of surface receptors, ultimately creating changes in the focal adhesions [4]. While the ECM has the ability to alter cellular function, so too do cells have the ability to alter the ECM. In what is known as dynamic reciprocity, the cellular response to the ECM can often alter the state of the ECM. For example, cells may release matrix metalloproteases to break down an overly dense ECM to allow for their migration or proliferation [2,3]. Creating an ECM analogue that can replicate these physiologic cell-ECM interactions is extremely challenging, yet may be possible through the manipulation of the natural polymers which constitute the native ECM, as these polymers may present the normal signaling capabilities required by cells [1].

**Figure 1.** Diagram of cell-ECM interaction depicting integrin binding sites.



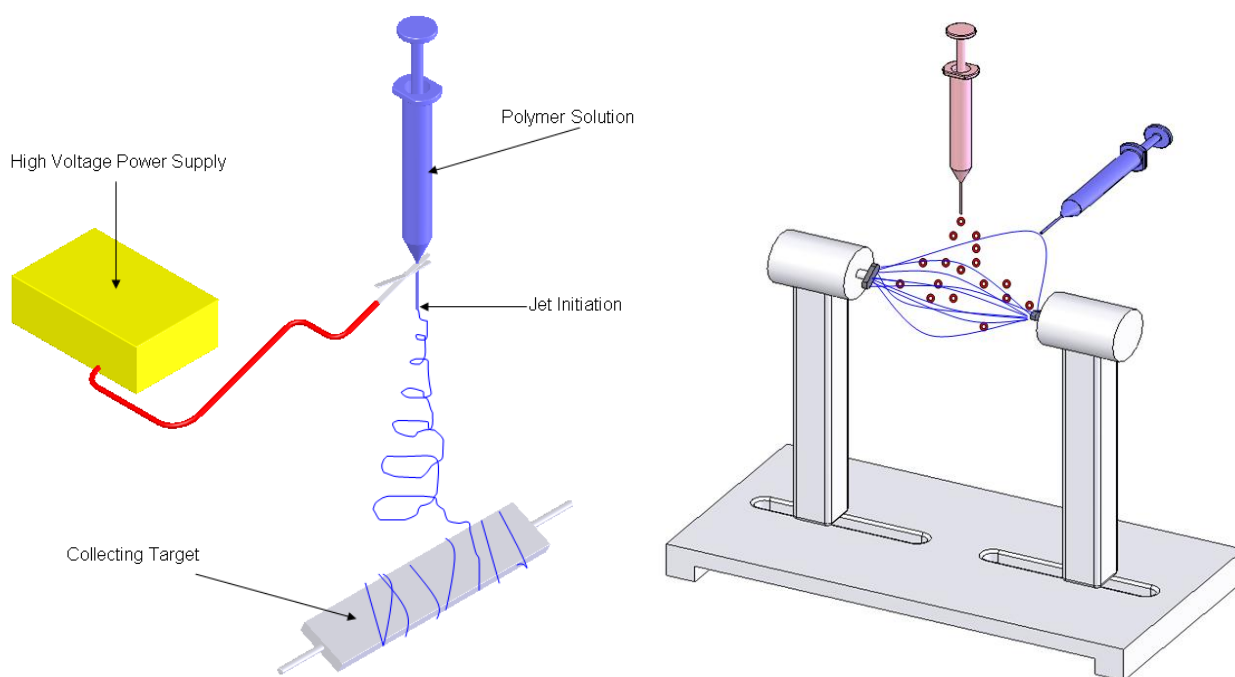
### 1.2. Electrospinning

Ideally, a tissue engineering scaffold should mimic both the form and functionality of the native ECM. However, due to its complexity and diversity, it is virtually impossible to manufacture an exact replica that can encompass all of the roles of the native ECM. To be successful as a tissue engineering scaffold, structures must: 1) have appropriate porosities to allow for cellular migration and penetration; 2) have sufficient surface area and the proper surface chemistry to promote cell adhesion, growth, migration, and differentiation; 3) a rate of degradation that closely matches the rate of native tissue regeneration to promote proper tissue ingrowth. In the past decade the process of electrospinning has become a widely used technique for the creation of ECM analogue structures for tissue engineering applications, in part because of its capacity to meet the three previously mentioned criteria. With its ability to rapidly create structures composed of nanoscale fibers closely resembling the architecture of the native ECM, this process has experienced a renaissance in recent years and continues to find new niches in the field of tissue engineering. This relatively simple process has proven to be highly adaptable, with the capacity for use with a number of natural and synthetic polymers, cost effectiveness, easy scale-up for large volume production, and the ability to provide users control over a number of processing parameters which can be modified to elicit fine control over the end-product's physical features [1,5-11].

In its simplest form the process of electrospinning requires little specialized equipment; all that is needed is a high voltage power supply, a grounded target, and a small diameter conductive capillary to emit a polymer solution in a controlled fashion (Figure 2). Polymers are dissolved in a solvent, everything from water to organic solvents such as hexafluoroisopropanol (HFP) depending upon the polymer, at a concentration high enough for polymer chain entanglement to occur. As solution viscosity increases with polymer concentration, the resulting fiber diameter increases linearly [1,12]. The polymer solution is fed through a capillary (typically a needle with a blunt end) at a constant flow

rate, with the polymer solution within the capillary charged to a high voltage potential opposite a grounded target to create a static electric field. When the electric field produces a force strong enough to overcome the surface tension of the polymer solution collected at the capillary tip, a Taylor cone forms. This Taylor cone is then stretched until a jet of polymer is drawn from the tip of the Taylor cone and attracted to the grounded collecting target. As the jet travels through the air towards the collecting target the solvent gradually evaporates, resulting in the collection of nano to micron scale fibers on the target. The charge from the fibers dissipates into the surrounding environment, and a non-woven fiber mat consisting of fibers 50 nm to 10  $\mu\text{m}$  in diameter is formed [1,5-11,13,14].

**Figure 2.** Schematic representation of a traditional electrospinning setup with a horizontal orientation (Left), and the simultaneous microintegration of cells and electrospun polymer using an airgap electrospinning setup (Right).



The ability for electrospinning to create tissue specific scaffolds arises from the adaptability of the process and the system control offered by a number of tunable processing parameters: solution properties (viscosity, elasticity, conductivity, and surface tension), processing conditions (voltage, capillary diameter, distance from capillary orifice to grounded target), and environmental conditions (temperature, humidity and static electricity) [6,9,10,15]. Each of the aforementioned parameters, separately or in conjunction with one another, can affect fiber deposition, fiber diameter, and scaffold porosity. By altering the orientation of the collected fibers (parallel alignment *versus* random fiber orientation), and/or the type of polymer(s) used (natural, synthetic, or blended) it is possible to significantly alter the mechanical properties of the scaffolds. In addition to altering the orientation of the fibers, variations in the micro- and macro-structures of the scaffolds themselves can be obtained for specific applications. For example, multilayering electrospinning results in a hierarchically ordered structure composed of different types of polymers, while multicomponent electrospinning, where multiple polymers are simultaneously electrospun together, forms a mixed fiber mesh [16]. Also, the

way the fibers are collected on the grounded target can influence the fiber orientation as well as scaffold fabrication. Some of the collection schemes currently used include a single ground, rotating single ground, dual bar, dual ring, single horizontal ring, electrospinning *in vitro* onto cells [15,17], electrospinning cells simultaneously with polymer (microintegration) [18], or using a rotating electrode airgap collection method [19]. The microintegration process developed by Stankus *et al.* [18] used the principle of electrospraying, a historical precursor to electrospinning, to disperse small charged droplets of cell suspension onto a target simultaneously collecting electrospun fibers. This process had no adverse effect on the cells, and proved to be a viable method for creating a cellularized structure primed for regeneration.

## 2. Electrospun Natural Polymers

### 2.1. Collagen

Collagen fibrils and their networks form the ECM for most hard and soft tissues (bone, cartilage, tendon, cornea, blood vessels, nerve, and skin) in the human body. Collagen plays a key role in maintaining the biologic and structural integrity of the ECM architecture, and presents different morphologies in different tissues. Additionally, it is highly dynamic, undergoing constant remodeling for proper physiological functions [1,2].

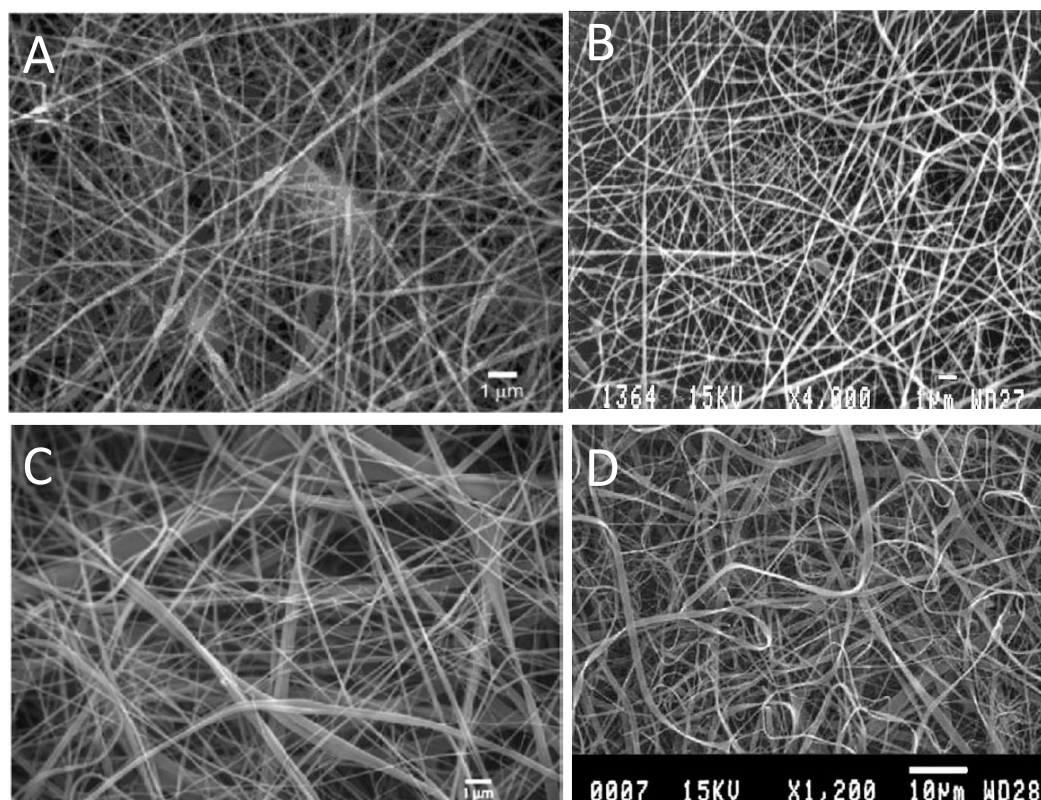
To date, 28 collagen types have been identified; with types I, II, III, V and XI involved in forming fibrillar structures. Collagen types I, II, III and V are the primary types that constitute the essential part of collagen in bone, cartilage, tendon, skin and muscle. While each collagen type is somewhat unique, all collagen molecules share a triple helical structure, and the presence of 4-hydroxyproline provides a distinctive marker for these molecules [3,4]. Collagen type I consists of two  $\alpha 1$  chains and one  $\alpha 2$  chain forming fibrils of 50 nm in diameter, and is present in bone, skin, dentin, cornea, blood vessels, fibrocartilage and tendon. Type II collagen is composed of three identical  $\alpha 1(\text{II})$  chains forming fibrils less than 80 nm in diameter and is found exclusively in cartilaginous tissues and is associated with water, proteoglycans, glycoproteins and noncollagenous proteins. Type III collagen is present in our skin as well as in ligaments, blood vessels and internal organs, and is composed of three  $\alpha 1(\text{III})$  chains resulting in fibrils ranging from 30 to 130 nm in diameter [5,6,9]. Type IV collagen, which is composed of  $\alpha 1(\text{IV})$  and  $\alpha 2(\text{IV})$  chains, is the major skeletal macromolecule of basement membrane in various tissues. The molecular organization of type V collagen, in terms of  $\alpha 1(\text{V})$ ,  $\alpha 2(\text{V})$ , and  $\alpha 3(\text{V})$  chain compositions, still remains to be clearly identified. Type V collagen has been identified in many tissues including blood vessel wall, synovium, corneal stroma, tendon, lung, bone, cartilage and skeletal muscle [20].

Aside from its widespread presence natively in the body, collagen has several properties that make it an attractive biomaterial for tissue engineering applications: low antigenicity, low inflammatory and cytotoxic responses, high water affinity, good cell compatibility, availability of various methods of isolation from a variety of sources, and biodegradability [4,8,11].

### 2.1.1. Electrospinning Collagen

In 2001, the electrospinning of collagen was first reported with the use of poly (ethylene oxide) (PEO) [21]. Since then, the work in our laboratory on the electrospinning and characterization of collagen types I [22], II [23], III [24] and IV [24] has demonstrated that the electrospinning process has the potential to produce collagen fibers that closely mimic, and at some point may even fully reproduce, the structural and biological properties of the natural collagen ECM (Figure 3). Transmission electron microscopy revealed the 67 nm characteristic D-repeat banding pattern on electrospun type I collagen, nearly identical to that seen natively in collagenous structures. Electrospun fibers of type I, II and III collagen dissolved in HFP have exhibited a linear relationship between polymer concentration and average fiber diameter, with the lower fiber size range falling within the 50–500 nm seen in native tissue. Electrospun type I collagen fibers have been fabricated with diameters ranging from 100 nm–5  $\mu\text{m}$ , with diameters of 20–200 nm for type II collagen, and fibers of 115–612 nm for collagen type III [1,9]. Our laboratory has also done some preliminary work on the electrospinning of collagen type IV from HFP, to form fibers with diameters ranging from 100 nm–2  $\mu\text{m}$  [1].

**Figure 3.** Scanning electron micrographs (SEM) of electrospun collagens type I (A), reprinted with permission from [22]. Copyright 2002 American Chemical Society. Collagen type II (B), reprinted from [23], copyright © 2003 by Sage Publications. Reprinted with permission of SAGE. Collagen type III (C) [24], and collagen type IV (D). Reprinted from [1], Copyright 2007, with permission from Elsevier. SEM scale bars = 1  $\mu\text{m}$  (A,B, and C) and 10  $\mu\text{m}$  (D).



As with other collagenous scaffolds, fabricated as gels or sponges, electrospun collagen lacks mechanical and structural stability upon hydration. In order to increase the strength of electrospun collagen, cross-linking with glutaraldehyde vapors, formaldehyde and epoxy compounds has been evaluated. Glutaraldehyde cross-linked collagen type I scaffold showed tensile strength close to several commercially available wound care products such as Beschitin<sup>®</sup> and Resolute<sup>®</sup> LT [5]. Lower porosity and reduced numbers of keratinocytes and fibroblasts (FB) were observed on the cross-linked scaffold nanofibers as compared to matrices treated with collagen type I.

This approach however, leads to an enhanced risk of cytotoxicity and calcification when used *in vivo*. In 2007, our laboratory published a technique for the cross-linking of electrospun collagen, while preventing any cytotoxic effects, imparting desirable mechanical properties, and maintaining the nanofibrous structure, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in ethanol [25]. Shih *et al.* also studied the morphology, growth, adhesion, motility, and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells on EDC cross-linked electrospun collagen fibrous mats [14]. Electrospinning of collagen type I using buffer/ethanol solutions has been successfully accomplished by Dong *et al.* The scaffolds were cross-linked using EDC, and the authors demonstrated that the triple helical structure of collagen was preserved and that aligned fibers could be obtained by using this method [26].

### 2.1.2. Electrospinning Collagen and Natural Polymer Blends

Blends of collagen and glycosaminoglycans (GAG) have been utilized extensively for dermal regeneration. Chondroitin sulfate has been added to collagen type I for dermal regeneration templates and aggrecan (chondroitin sulfate/dermatan sulfate/keratin sulfate) to collagen type II for articular cartilage tissue engineering [27]. Zhong *et al.* developed blended scaffolds of collagen and GAG, cross-linked with glutaraldehyde. These scaffolds allowed for increased proliferation of rabbit conjunctiva FBs [28]. Our group has electrospun collagen-GAG blends by adding the GAG directly to the electrospinning solution or by dissolving the GAG at higher concentrations in distilled water prior to blending with the electrospinning solution. Preliminary results indicated that the dimensions of the fibers decreased relative to pure collagen scaffold [1].

Nanofibrous biocomposite scaffolds of type I collagen and nanohydroxyapatite (nanoHA) of varying compositions (wt%) were prepared by Thomas *et al.* for bone tissue engineering applications [29]. The surface roughness, fiber diameter and the tensile strength of the scaffold was increased with the presence of nanoHA. Chemical cross-linking with glutaraldehyde further enhanced the mechanical properties of the scaffold.

Chen *et al.* fabricated nanocomposite scaffolds of collagen, chitosan and PEO for wound dressing applications. The constructs showed no cytotoxicity toward growth of 3T3 FBs and had good *in vitro* biocompatibility [30]. In another study, collagen blended with chitosan showed both endothelial cell (EC) and smooth muscle cell (SMC) proliferation on or within the nanofibers [31]. Yeo *et al.* used collagen and SF to create two types of electrospun scaffolds, a blend scaffold and a hybrid scaffold. The blended scaffold was created by mixing the two polymer solutions prior to electrospinning, while the hybrid scaffold was electrospun simultaneously from two syringe needles [32]. They concluded that the hybrid matrix was better for wound dressing applications as cell attachment and the spreading



of normal human epidermal keratinocytes was better on the hybrid matrix as compared to the blend matrix.

### 2.1.3. Electrospinning Collagen and Synthetic Polymer Blends

Blending collagen with other natural and/or synthetic polymers has enabled tissue engineers to fine tune the desired properties of the electrospun scaffolds. Collagen acts as an adhesion protein in the native ECM, enhancing cell attachment and proliferation through specific interactions between domains in collagen molecules and integrin receptors in the cell membrane (*i.e.*, RGD (Arg-Gly-Asp)). Thus, the presence of collagen in a synthetic polymer scaffold is intended to impart biocompatibility and bioactivity to the scaffolds, with the synthetic polymer providing mechanical integrity to the structure. Other approaches for introducing proteins into nanofibrous structures, such as coating or grafting, lead to slow mass transfer of the proteins into the three-dimensional porous materials. Electrospinning of a blended collagen mixture is not only simpler, but also avoids the slow mass transfer process and uses lesser amounts of chemical reagents. Moreover, the existence of collagen on the surface and inside the structure provides sustained cell recognition signals with polymer degradation, which is crucial for cell function and development [33].

Scaffolds composed of collagen and synthetic polymers have been widely used for cardiovascular tissue engineering applications. In a study done by He *et al.* [33], it was demonstrated that collagen type I blended poly (L-lactic acid)-co-poly( $\epsilon$ -caprolactone) [(PLLA-CL), 70:30] biohybrid scaffolds could enhance the viability, spreading and attachment of human coronary artery endothelial cells (HCAECs) and preserve their phenotype. A similar approach was used by Kwon and Matsuda [34] to combine PLLA-CL with collagen type I. Scaffolds blended with collagen were found to increase the attachment, spreading and proliferation of human umbilical vein endothelial cells (HUVECs).

Stitzel *et al.* fabricated a prototypic vascular graft using biodegradable poly (lactic acid) (PLA) fibrous meshes and collagen type I fibers. Collagen fibers were wound using a novel winding apparatus with variable pitch to mimic the ECM structure in an artery. PLA matrix was spun around this fiber for SMC seeding and to hold the fibers in place during tissue regeneration. Confluent layers of human aortic SMCs were observed in the luminal and external surfaces of the vascular graft. The alignment of the SMCs along the direction of the collagen fibers was also observed [35].

Kidoki *et al.* created a tri-layered electrospun mesh composed of type I collagen, styrenated gelatin (ST-gelatin), and segmented polyurethane (SPU) in which individual fiber meshes were deposited layer by layer by sequential electrospinning. Confocal laser scanning micrographs of the scaffolds revealed the SPU, ST-gelatin, and collagen layers to be segregated and hierarchically positioned in the order of the vertical direction of the meshes. The authors speculated that such hierarchically designed artificial grafts may provide compliance matching that of native arteries, cell and tissue ingrowth, and transient antithrombogenicity in the early phase of implantation [16].

Polydioxanone (PDO) is a highly crystalline polymer, commonly used as a commercially available suture that has been shown to exhibit excellent mechanical properties, elasticity, shape memory, a low inflammatory response, and a slower rate of degradation than other resorbable suture materials [36]. Our laboratory has created electrospun blended scaffolds of PDO and collagen types I and III in ratios of 100:0, 90:10, 80:20, 70:30 and 50:50 (PDO:collagen by volume). The addition of collagen to PDO

resulted in average fiber diameter measurements comparable to those seen in the native ECM fibers (210–340 nm), surprisingly with no significant difference in fiber diameters with the addition of increasing amounts of collagen. The basic mechanical properties of the hydrated electrospun blends of PDO and collagen were on the same order as those of clinically used autologous and synthetic vascular grafts; peak stress values in the range of 4.6–6.7 MPa were achieved, with an average modulus of 7.6–18.0 MPa, and an average strain at break of 56.6–186.4%. Preliminary *in vitro* cell culture with human dermal FBs demonstrated favorable cellular interactions on all constructs containing collagen, with prominent cell migration into the thickness of the scaffolds compared to simple surface spreading with no penetration on pure PDO scaffolds [24].

Polycaprolactone (PCL) is an inexpensive, bioresorbable polymer with excellent mechanical properties, lack of toxicity and slow degradation time [37,38]. Lee *et al.* developed PCL/collagen type I composite scaffolds with the ability to resist high degrees of pressurized flow over long durations, while still providing a favorable environment for the growth of vascular cells [37]. The burst pressure of the composite scaffolds ( $4912 \pm 155$  mmHg) was much larger than that of the pure PCL scaffolds ( $914 \pm 130$  mmHg), and it also exceeded the burst strength of native vessels. The scaffolds were also found to be conducive to bovine EC and SMC attachment and proliferation. These PCL/collagen electrospun scaffolds were also investigated for their *in vivo* stability in a rabbit aortoiliac bypass model. It was observed that endothelialized grafts resisted adherence of platelets when exposed to blood. Also, it was demonstrated by ultrasonography that these scaffolds were able to retain their structural integrity over 1 month of implantation. Moreover, these scaffolds continued to maintain biomechanical strength at retrieval that was comparable to native artery [38].

Blended nanofibers of PCL and collagen types I and III were fabricated with average fiber diameters of 210–225 nm, tensile modulus of 18 MPa and a tensile strength of 7.79 MPa in a study by Venugopal *et al.* [39]. These scaffolds supported growth, proliferation and migration of SMCs inside the matrices. Cell proliferation was significantly increased up to 63%, 73% and 82% in PCL/collagen nanofiber scaffolds compared to PCL nanofiber scaffolds after 2, 4 and 6 days, respectively.

Unidirectionally oriented electrospun PCL/collagen nanofibers were designed for use as a scaffold system for implantable engineered muscle. *In vitro* studies revealed that the aligned nanofibers significantly induced human skeletal muscle cell alignment and myotube formation as compared to randomly oriented nanofibers [40]. Aligned PCL/collagen scaffold was also fabricated by Schnell *et al.* for use as artificial nerve implants. It was found that Schwann cell migration, neurite orientation, and process formation of Schwann cells, FBs and olfactory ensheathing cells was improved on PCL/collagen nanofibers as compared to pure PCL scaffolds. Analysis of isolated sensory neurons showed significantly better axonal guidance by the PCL/collagen scaffold [41].

## 2.2. Gelatin

Gelatin, a structurally similar derivative of collagen, is acquired by denaturing the triple-helix structure of collagen [1,42]. Gelatin, which is typically derived from bovine or porcine skin, is an attractive polymer for tissue engineering because of its biological origin and similarity to collagen. The average fiber diameters of electrospun gelatin, as well as scaffold mechanical properties, have been found to be similar to those of electrospun collagen fibers. For both polymers, fibers can be

scaled down to 200–500 nm in diameter, and tensile strengths are around 8–12 MPa, with ultimate elongations of 0.08–0.1 mm/mm [43]. However, gelatin fibers exhibit higher tensile moduli than collagen fibers. Gelatin also exhibits excellent biodegradability, non-antigenicity, and cost efficiency; similar characteristics to electrospun collagen [44–46]. One of the major drawbacks of gelatin is that it dissolves as a colloidal sol at temperatures at or above 37 °C, and gels near room temperature. As such, electrospun gelatin is often cross-linked or combined with synthetic polymers in order to maintain a fibrous structure. Several studies have demonstrated the use of different cross-linkers to reduce the limitations mentioned above, including 1,6-diisocyanatohexane (HMDI), glutaraldehyde, genipin, and glycerinaldehydes [47–50]. Various studies have also looked at electrospinning gelatin nanofibers from different solutions, including the organic solvents HFP and trifluoroethanol (TFE), as well as formic acid, acetic acid, ethyl acetate and water. Due to its similarity to collagen and its bioactivity, electrospinning of gelatin and gelatin/synthetic polymer blended scaffolds have been gaining interest for tissue engineering applications, and several studies have detailed their efficacy [43–46,51–58].

Electrospun gelatin scaffolds have been used for various applications such as wound healing [59,60], nerve [52,61,62], dental [49], bone [48,50,57,63], dermal tissue engineering applications [64,65] and vascular grafts [66]. Much of the work published on electrospun gelatin scaffolds has been directed towards its use in cardiac tissue engineering applications. Li *et al.* [67] electrospun gelatin with a conductive polymer, polyaniline. Pure gelatin scaffolds (8% w/v) were fabricated with fiber diameters of 800 nm, tensile strength of 5.7 MPa, modulus of 499 MPa, and an elongation of 96%. The addition of polyaniline (3% w/v at ratios of 15:85, 30:70, 45:55, and 60:40 polyaniline:gelatin) at increasing concentrations caused a decrease in the fiber diameter and an increase in the mechanical integrity of the scaffolds. Cell culture analysis done with H9c2 rat cardiac myoblasts resulted in attachment, spreading, migration and proliferation to confluence on the scaffolds. In a similar study, PLGA, gelatin, and elastin were co-electrospun at 10%, 8%, and 20% (w/v) respectively into scaffolds and exhibited increased mechanical properties, with moduli of 122–254 MPa. These scaffolds also facilitated excellent cell proliferation, morphology and penetration of H9c2 rat cardiac myoblasts and neonatal rat bone marrow stromal cells demonstrating their ability to be used in cardiovascular tissue engineering applications [68].

Gelatin has also been electrospun with PCL in different combinations to create scaffolds that avoid the need for post-fabrication cross-linking [44]. In a study done by Heydarkhan-Hagvall *et al.*, electrospun gelatin (5, 7, and 10% w/v) and PCL (1, 5, 7, and 10% w/v) scaffolds exhibited fibers ranging from 640–880 nm in diameter depending on the amount of gelatin and PCL, where an increase in the percentage of gelatin and PCL resulted in larger fiber diameters. Tensile strength was measured to be 6.14–11.17 MPa, with a Young's modulus of 57–138 MPa. In the same study, PCL was also blended with collagen, elastin, and gelatin biopolymers. It was observed that with increasing protein and polymer concentrations, fiber size increased proportionally but pore size decreased. In terms of mechanical properties, electrospun gelatin/PCL scaffolds displayed a higher tensile strength when compared to collagen/elastin/PCL scaffolds. All hybrid scaffolds were seeded with adipose-derived stem cells. SEM and nuclei staining of cell-seeded scaffolds showed complete cell attachment to the surfaces of both gelatin/PCL and collagen/elastin/PCL hybrid scaffolds, but cell penetration into the scaffold was primarily seen in the gelatin/PCL hybrid scaffold [46].

Recently, interesting alternatives to bovine and porcine derived gelatin, which may prove to be beneficial as a scaffold for tissue engineering, have been published. Gelatin extracted from Nile tilapia was electrospun from an acetic acid solvent or a formic acid solvent and resulted in fiber diameters ranging from 161–761 nm for acetic acid and 109–302 nm for formic acid. Mechanical properties for the pure gelatin fiber mats included a tensile strength of 2.4–4.2 MPa, Young's modulus in the range of 118–194 MPa, and the elongation of 3.3–37.6%. Gelatin scaffolds were cross-linked, which caused the mats to become stiffer, with tensile strength increasing to 4.9–10.6 MPa, Young's modulus being 366–570 MPa, and the elongation ranging from 3.3–25.7% [53]. Gelatin has also recently been extracted from Channel catfish skin and electrospun from a formic acid solvent. The electrospun gelatin scaffolds exhibited fiber diameters ranging from 184–485 nm. Mechanical properties were similar to those of the gelatin extracted from Nile tilapia, with a tensile strength of 2.32 MPa, Young's modulus of 127.4 MPa, and elongation of 10.6% [69].

### 2.3. Elastin

Elastin is a key structural protein found in the native ECM of connective tissues where elasticity and recoil are critical parameters. Physiologically, elastin constitutes the walls of arteries and veins, ligaments, lung parenchyma, skin, and intestines [1,70]. A chemically inert, highly insoluble polymer, elastin is composed of several covalently cross-linked molecules of its precursor, tropoelastin, a 67-kDa soluble, non-glycosylated, highly hydrophobic protein [70,71]. Elastogenesis, the formation of native elastin, starts with the synthesis of tropoelastin inside the cell, after which elastin binding protein (EBP) transports these tropoelastin molecules to specific sites at the cell-surface, and prevents its premature intracellular aggregation and proteolytic degradation. Once tropoelastin reaches the cell surface, galactosugars on the microfibrils bind to the lectin-binding site of the EBP, excreting tropoelastin into the extracellular space. Once the N-terminal part of the microfibrillar-associated glycoprotein is aligned with the C-terminal end of tropoelastin, the  $\text{Cu}^{+2}$ -dependent enzyme lysyl oxidase deaminates and oxidizes the lysyl residues to allysine [11,71,72]. Cross-links are then formed by the reactions of these allysines with themselves or with an unmodified lysine, which leads to the polymerization of soluble tropoelastin to insoluble elastin.

Elastin has become increasingly popular as a biomaterial for various tissue engineering applications. As elastin synthesis naturally occurs in FBs, vascular SMC, ECs, and chondrocytes, it also has been shown to modulate the cellular physiology, influencing signaling, chemotaxis, proliferation, and protease release of these same cells, as well as monocytes, macrophages, neutrophils, and lymphocytes [72]. The incorporation of elastin as a biomaterial has been used in several forms, including insoluble elastin in autografts, allografts, xenografts, decellularized ECM, and in purified elastin forms where the insoluble elastin has been hydrolyzed to a soluble form. Repeated elastin-like sequences have been produced by synthetic or recombinant means [73], and even recombinant tropoelastin or tropoelastin fragments have been used as biomaterials [74,75]. The use of soluble elastin is advantageous over its insoluble form due to the straightforwardness in the handling and analysis of the material. When used as a scaffold *in vivo*, soluble elastin exhibits no signs of calcification, a problem with insoluble elastin scaffolds. Soluble elastin scaffolds have also been

shown to exert positive biological effects on a variety of cell types, including increasing angiogenesis and elastic fiber synthesis [75].

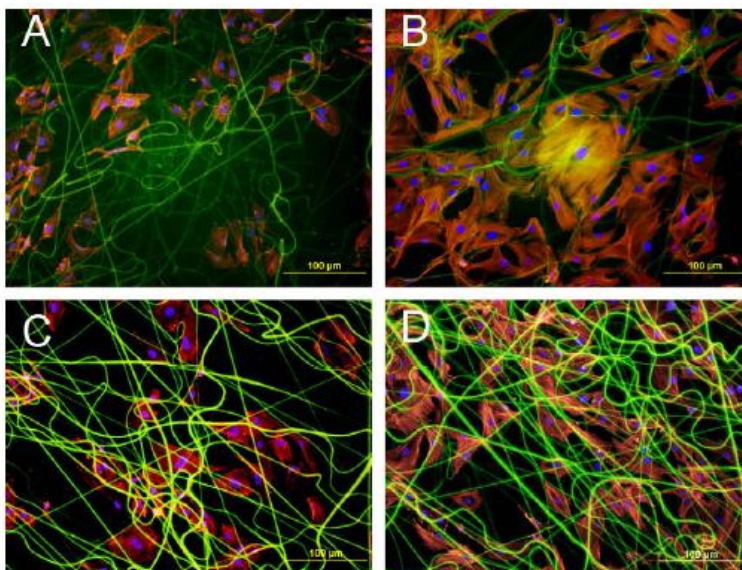
### 2.3.1. Electrospinning Elastin

As mentioned previously, elastin naturally constitutes the walls of many tissues, so to be able to mimic this fibrous structure in tissue engineering applications is particularly favorable. Being one of the major constituents of the vessel wall, much of the work on electrospun elastin is for vascular graft applications, however, other studies have used elastin for applications such as skin [76], heart valves [77], and elastic cartilage [78].

Alpha-elastin and tropoelastin have previously been electrospun by Li *et al.* [43]. The proteins were electrospun from HFP, forming fibers that appeared flattened with “quasi-elastic” wave-like patterns with diameters in the nano- and micron scale range, depending on the polymer delivery rate. Like the majority of all electrospun polymers, there was a linear correlation between the concentration of the electrospinning solution and the fiber diameter. In addition to fiber diameter, mechanical properties were determined with microtensile testing, and biocompatibility was analyzed by seeding scaffolds with human embryonic palatal mesenchymal (HEPM) cells. Tropoelastin proved to be more elastic than solubilized elastin, and both scaffolds supported cell attachment, migration and proliferation. After 6 days, there were significantly more cells on both alpha-elastin and tropoelastin scaffolds than tissue culture plastic controls. More recently, Nivison-Smith *et al.* electrospun tropoelastin from HFP and cross-linked the scaffolds to form synthetic elastin microfibrillar constructs [79]. The fibers demonstrated a ribbon-like appearance, with diameters ranging from 0.9–2.7  $\mu\text{m}$ . The biocompatibility of the cross-linked and non-cross-linked structures was analyzed by characterizing cell growth, attachment, and morphology of human dermal FBs, HUVECs and human coronary artery smooth muscle cells (HCASMC) that were seeded on the scaffolds. Cells were found to attach and grow on the seeded surface of the cross-linked scaffolds, and no effects from the different cross-linking methods were seen on cell morphology and proliferation (Figure 4). The study demonstrated that human tropoelastin retained its secondary structure after electrospinning and its functional ability to associate by coacervation.

Huang *et al.* were able to electrospin a synthetically based elastin-mimetic. Based on the repeated elastomeric peptide sequence of natural elastin, the synthetic protein was electrospun out of ultrafiltered grade, distilled, deionized water, and was able to produce fibers that were 300–400 nm in diameter [73]. Uniaxial stress-strain properties of the uncross-linked scaffolds were also characterized and ultimate tensile strength was found to be 35 MPa, and modulus was found to be 1.8 GPa.

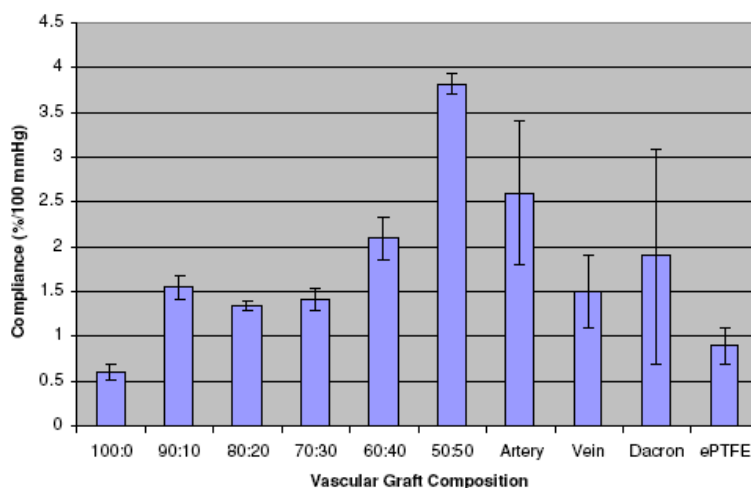
**Figure 4.** Fluorescence microscopy images of DAPI and rhodamine phalloidin staining of (A and B) HUVECS and (C and D) HCASMCs on HMDI cross-linked synthetic elastin microfibers (A and C) and glutaraldehyde cross-linked microfibers (B and D) after 24 hours. Reprinted from [79], Copyright 2010, with permission from Elsevier.



### 2.3.2. Electrospinning Elastin and Synthetic Polymer Blends

Similar to other electrospun natural polymers, pure electrospun soluble elastin exhibits poor mechanical properties, and has been shown to dissolve in water instantaneously if not cross-linked. One solution to this problem is to blend it with synthetic polymers, which not only help to maintain the structural integrity of the scaffold, but also retains the elastin within the scaffold [80]. McClure *et al.* found that elastin blended with PDO dissolves over time, but with increasing amounts of synthetic polymer, the time to dissolve elastin from the scaffolds increases. In addition, scaffolds with a large percentage of elastin experienced instantaneous weight loss, whereas scaffolds with increasing amounts of PDO lost weight at a much slower rate. As part of that study, the elastin and PDO scaffolds were cross-linked with either EDC or genipin, both which allowed the elastin scaffolds to retain their original weight throughout the experiment, and only slightly altered their mechanical properties [80]. Other cross-linking agents that have been used involving soluble elastin include glutaraldehyde [81], EDC [80,82], or HMDI [43,79]. In a different study performed by the authors of this manuscript, compliance was determined for combinations of electrospun PDO and elastin grafts. Under dynamic conditions which mimic physiological parameters, it was determined that certain PDO:elastin blends, specifically 60:40 and 50:50 v/v, matched closely the compliance of the native artery (Figure 5) [83].

**Figure 5.** Compliance determined for 120/80 mmHg pressure level for native artery and vein, current synthetic vascular grafts, and electrospun PDO:Elastin blends. Reprinted with permission from IOP Publishing Ltd. [83].



In a study by Smith *et al.* dual-layered PDO:elastin tubes were wound with either zero, one or two 6-0 sutures between the layers [84]. Burst strength and compliance of these grafts was analyzed, and displayed compliance similar to that of the native artery. This study also demonstrated the ability to tailor the mechanical properties of the electrospun PDO:elastin scaffold, a major benefit for various tissue engineering applications.

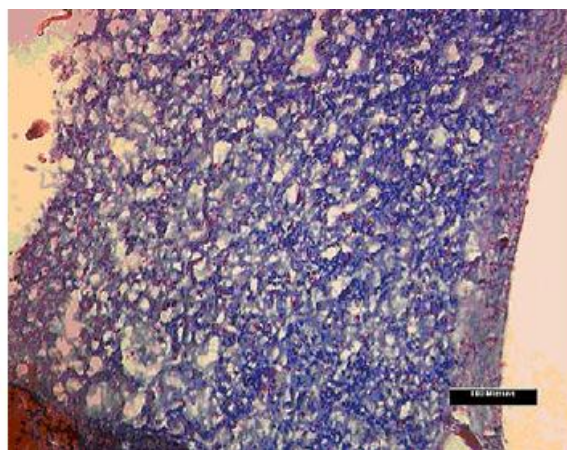
Thomas *et al.* have created an electrospun nanofibrous trilayered tubular conduit of PDO, gelatin and elastin containing spatially designed layers of elastin:gelatin, PDO:elastin:gelatin, and PDO:gelatin. The trilayered construct was designed to represent the intima, media and adventitia of an artery. Incorporating more elastin to PDO and gelatin solutions caused the fiber diameter to decrease from 850–900 nm to 300–450 nm [85]. In agreement with the results of various other studies on natural and synthetic polymer blended scaffolds, with the addition of more natural proteins, the mechanical integrity and degradation time of the scaffold decreased. A different trilayered electrospun construct was composed of elastin:gelatin, Maxon:elastin:gelatin, and Maxon:gelatin [86]. Similar to the trilayered structure with PDO, mechanical testing and degradation time decreased with increasing natural protein components. In both cases, slightly changing the parameters in one layer subsequently changed the overall material properties of the grafts.

### 2.3.3. Electrospinning Collagen and Elastin

Collagen and elastin are often electrospun together to produce tissue engineering scaffolds for vascular graft applications, as they are the two main constituents of native blood vessel. Boland *et al.* [81], in an attempt to create a biomimicking layered vascular structure, electrospun an 80/20 collagen type I/elastin tube onto a 4 mm diameter mandrel. This tube was subsequently seeded with both FBs and SMCs. Another tubular scaffold of 30/70 collagen type I/elastin was electrospun onto a 2 mm diameter mandrel. This electrospun scaffold was then inserted into the 4 mm diameter scaffold and the lumen was filled with a SMC suspension. After 3 days in culture, a suspension of HUVECs was injected into the lumen, and the entire construct was cultured for 2 more days. Histological examination revealed

complete cellular infiltration into the three-layered construct after 21 days. The artificial intima was covered by morphologically mature ECs, and SMCs were present throughout the media and had begun to align circumferentially around the axis of the scaffold. The FBs and SMCs in the adventitia created a dense population throughout the outermost wall; Figure 6 demonstrates the degree of SMC penetration and scaffold remodeling seen in the 500  $\mu\text{m}$  thick collagen type I/elastin structure after 21 days in culture.

**Figure 6.** Electrospun collagen/elastin scaffold showing extensive SMC infiltration, and a highly dense distribution of cells across the entire cross-section of the scaffold. Reprinted with permission from *Frontiers in Bioscience* [81].



To avoid the use of organic solvents, Buttafuoco *et al.* electrospun collagen type I and soluble elastin from aqueous acidic solutions [82]. With the addition of PEO and sodium chloride (NaCl) to ensure homogenous fibers, electrospun scaffolds exhibited fibers with diameters ranging from 220–600 nm. To stabilize the scaffolds, meshes were cross-linked in EDC in the presence of N-hydroxysuccinimide (NHS) in ethanol/water (70% v/v), and did not appear to dissolve after 14 days. As expected, these scaffolds proved to be biocompatible, as histological evaluation confirmed a confluent multi-layer of SMCs on the surface after 14 days.

Synthetic polymers have also been used in combination with collagen and elastin to create scaffolds with desirable biological and mechanical properties. Stitzel *et al.* [87] created nanofibrous scaffolds using a mixture of collagen type I, elastin and PLGA. Scaffolds were found to be conducive to SMC and EC growth. *In vivo* implantation studies in mice revealed no systemic or neurological toxicity. In a similar study, collagen and elastin were mixed with PLGA, PLLA, PCL and PLCL to create a tubular scaffold [88]. To evaluate dimensional stability, each tubular construct was incubated in culture medium, and the inner diameter of each scaffold was measured over a 2 month time period. The collagen:elastin scaffold without the addition of a synthetic polymer collapsed in culture after only 4 days of incubation, while collagen:elastin:PLGA and collagen:elastin:PLCL scaffolds had only reduced to 39.8% and 25.8% of their initial diameters after 2 months, respectively. Collagen:elastin:PLLA and collagen:elastin:PCL scaffolds maintained their original diameters over the 2 months without contraction or swelling, and had only reduced to 90.1% and 87.4% of their initial diameters, respectively. El-Kurdi *et al.* electrospun a poly(ester urethane) urea, collagen and elastin



blend from HFP onto freshly excised porcine internal jugular vein segments to further enhance the structural integrity of the vein [89]. Histological and SEM images verified a strong attachment of the polymer wrap to the adventitial surface of the vein. The polymer wrap proved to be efficient in adding structural support, as electrospun porcine internal jugular veins appeared to be less stiff than sham controls when exposed to arterial conditions for 24 hours (pulsatile pressure of 120/80 mmHg and a mean perfusate flow of 100 mL/min). In addition, as confirmed by a live/dead assay, the electrospinning process had no deleterious effects on tissue viability [89].

#### 2.4. Fibrinogen

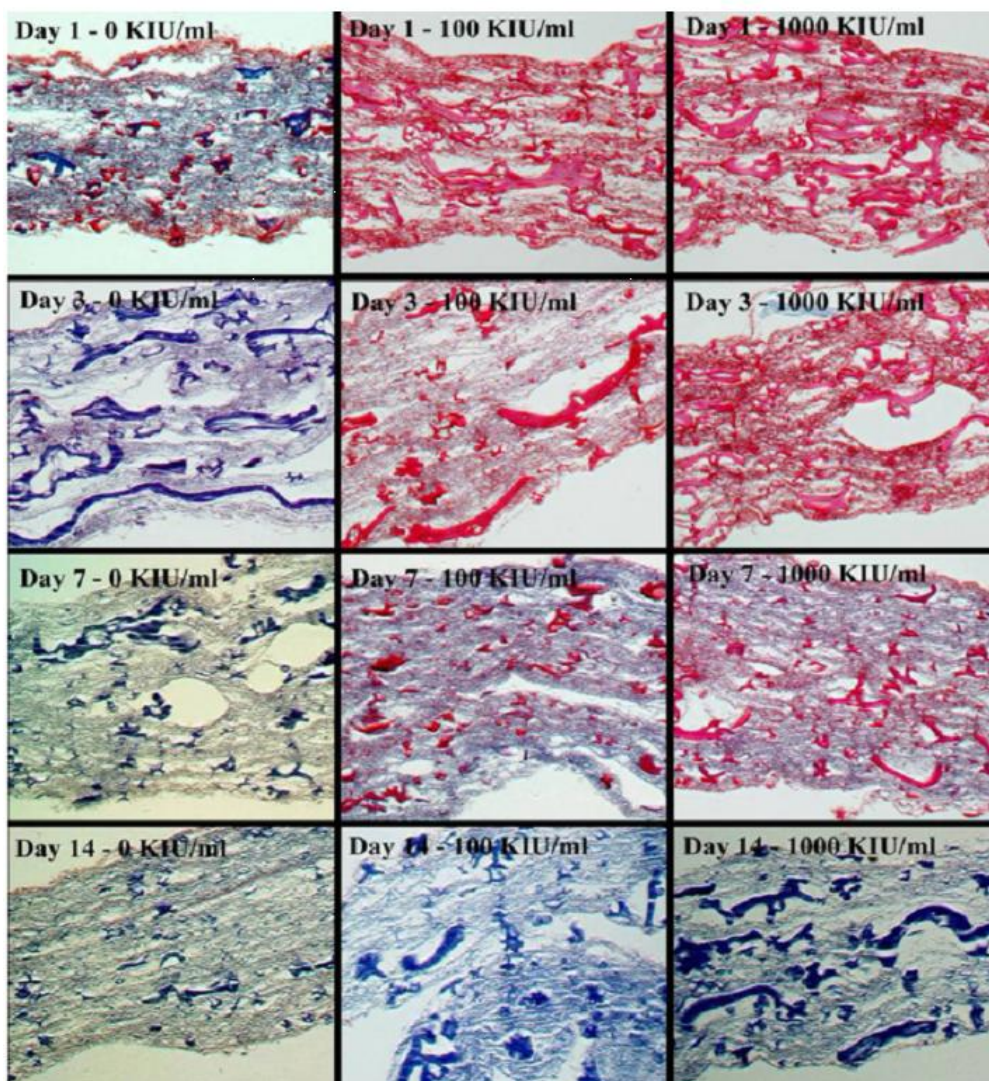
Fibrinogen is a 340 kDa glycoprotein comprised of a pair of three polypeptide chains: 2A $\alpha$ , 2B $\beta$ , and 2 $\gamma$ . Fibrinogen is synthesized by the liver, is freely circulating in the bloodstream, and plays a major role in hemostasis. During blood coagulation, fibrinogen is cleaved by thrombin converting it to fibrin. Fibrin spontaneously aggregates to form fibrin protofibrils which in turn aggregate laterally into larger fibers that branch to form a three-dimensional network. This results in the formation of a loosely assembled clot, subsequently stabilized by covalent cross-links created by plasma transglutaminase (factor XIIIa). This stable clot not only plays a haemostatic role, but also serves as an initial scaffold for tissue regeneration, serving as a platform for cell migration and proliferation [90-96].

As an important protein to the coagulation cascade, fibrinogen is commonly thought of as a wound dressing or haemostatic agent and not as a scaffold for tissue engineering. Fibrinogen, as a tissue engineering scaffold, has the potential to go beyond its primary role in clotting as it is a protein with the capacity to bind a wide variety of molecules. Fibrinogen contains integrin binding sites (*i.e.*, RGD) which commonly bind FBs and ECs, and has also been shown to bind with high affinity to fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and several other cytokines [94,97-99]. The presence of these functional growth factors could play a large role in promoting angiogenesis, a critical aspect in providing nutrients to an engineered scaffold for regeneration, as well as enhancing cell chemotaxis and mitogenesis at an implant site.

##### 2.4.1. Electrospinning Fibrinogen

The electrospinning of fibrinogen to develop nanofibrous tissue engineering scaffolds, wound dressings, and hemostatic products was first published by Wnek *et al.* [100]. Since then, electrospun fibrinogen scaffolds have been demonstrated as tissue engineering scaffolds with great potential. Preliminary *in vitro* cell culture with neonatal rat cardiac FBs proved electrospun fibrinogen scaffolds to be extremely bioactive, with the FBs readily migrating through the scaffolds and depositing native collagen [101]. In a similar study with human bladder smooth muscle cells (hBSMs), electrospun fibrinogen scaffolds demonstrated the ability to be degraded and remodeled over a short time course [102]. The rate of scaffold remodeling was so rapid, that seeded scaffolds were cultured in media containing aprotinin, a proteolytic enzyme inhibitor, at varying concentrations (0, 100, and 1,000 KIU/mL) to reduce the rate of scaffold degradation. As such, it was demonstrated that scaffold remodeling and collagen matrix production were inversely related to the aprotinin concentration, with nearly complete remodeling taking place after 14 days in even the highest aprotinin concentrations (Figure 7).

**Figure 7.** Optical micrographs of trichrome histology demonstrated the remodeling of fibrinogen and the production of new collagen matrix in electrospun fibrinogen scaffolds seeded with hBSMs. Scaffolds contained increasing amounts of aprotinin from left to right. Images at 20 $\times$ . Reproduced with permission from IOP Publishing Ltd. [102].

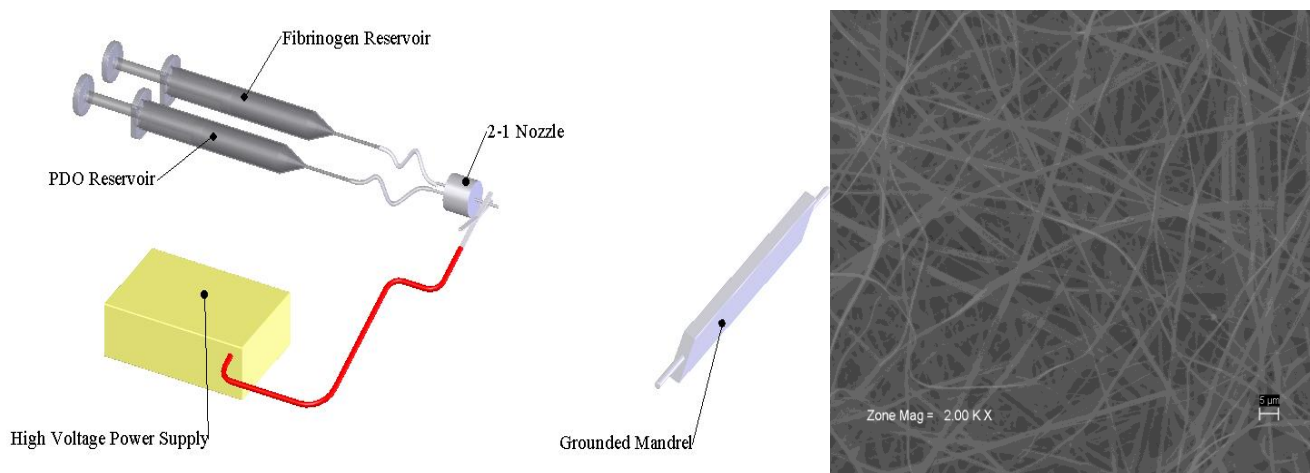


The mechanical properties of individual, electrospun fibrinogen fibers have been evaluated [103] and compared to the mechanical properties of wet, non-woven mats made from randomly oriented electrospun fibrinogen fibers [104]. Similarities were observed in the extensibilities of the individual fibers and the mats. However, both the peak stress and the modulus of elasticity of the mats were approximately an order of magnitude lower than the single fibers, suggesting that amongst other factors, the mechanical properties of the mats are a complex combination of the mechanical properties of the individual fibers making up the mats [103].

Similar to other natural polymers, electrospun fibrinogen lacks the mechanical integrity to serve as a tissue engineering scaffold on its own for long periods of time [104]. Using a novel 2-1 nozzle setup, solutions of fibrinogen and PDO were combined only at the Taylor cone to create a blended structure of the two polymers (Figure 8) [105]. These fibrinogen-based blends contained 0, 10, 20, 40, and 50% PDO by volume, and scaffolds were uniaxially tested to failure. The modulus of

elasticity (0.37–4.74 MPa) and peak stress (0.47–1.90 MPa) data demonstrated a linear increase from 0–50% PDO concentration. When seeded with hBSMs scaffold bioactivity was not adversely affected over the range of PDO concentrations, as collagen matrix production values were not different after 7 days in culture.

**Figure 8.** Electrospinning setup of fibrinogen and PDO scaffolds using a novel 2-1 nozzle (Left) and SEM of a 50:50 PDO:fibrinogen blended scaffold. Image at 2000 $\times$ , scale bar = 5  $\mu\text{m}$ .



Cross-linking of fibrinogen scaffolds has also been used to increase their mechanical strength and slow their rate of degradation. The authors of this review [106] have used both EDC and genipin to successfully alter the properties of electrospun fibrinogen, with cross-linked scaffolds remaining mechanically viable after 14 days in culture compared to complete loss of mechanical integrity after 7 days for non-cross-linked scaffolds. The degree to which these scaffolds were cross-linked decreased the rate of scaffold remodeling, with little new collagen matrix visible in Masson's trichrome histological stains after 21 days in culture with FBs. However, this study did demonstrate that through cross-linking it is possible to tailor the mechanical properties and rates of degradation of a highly bioactive natural polymer scaffold; the higher the degree of cross-linking the longer the scaffold will remain mechanically viable.

### 2.5. Silk Fibroin

Silk is a versatile biomaterial with significant crystallinity, high elasticity, strength and toughness, and resistance to failure in compression (even compared to Kevlar). SF has been used in textiles for centuries, and more recently has been used for medical and tissue engineering due to its high tensile strength and biocompatibility. The natural silk fiber consists of two cores of fibroin covered with a layer of sericin [107]. In nature sericin acts like an adhesive that is meant to help maintain the structure of the cocoon [107], however, in terms of using silk as a biomaterial, sericin can cause an adverse immune response if implanted in the body [108]. The gummy sericin protein is easily removed by boiling silkworm cocoons in water with salts.

SF consists of a heavy and light chain (350 kDa and 25 kDa), that are linked together by a disulfide bond [109]. Hydrophobic interactions cause the protein's random coil configuration to change to a  $\beta$ -sheet formation, which is responsible for the protein's tensile strength [110]. The combination of the  $\beta$ -sheet crystals, the interphase between the crystals, the semi-crystalline regions and the shear alignment of the molecular chains are the foundation for silk's unique mechanical properties. While the highly organized  $\beta$ -sheet regions of the protein provide the tensile integrity, the semi-crystalline regions are the basis for the protein's elasticity [111]. The  $\beta$ -sheet structure affects the tensile properties, degradation rate and elasticity of the scaffold, so the tailoring of these properties can be done in part with the cross-linking process. Methanol treatment is a widely used process to induce  $\beta$ -sheet formation although it does not transform all molecular regions. Ethanol, EDC, glutaraldehyde, and genipin cause the transition from random coils to  $\beta$ -sheet configurations of the scaffolds and is dependant on the length of time exposed to the solvent as well as the solvent concentration [112-116]. Studies have shown that regenerated silk fibers can hold their initial tensile integrity for 21 days under immune deficient *in vitro* culture conditions [117]. Moreover, the solvent used for electrospinning can affect the  $\beta$ -sheet formation of the scaffold's secondary structure, which in turn can alter the mechanical properties. Formic acid, HFP and water have been used to electrospin silk scaffolds, and of those, water and formic acid seem to enhance the mechanical properties of the scaffolds [118,119].

SF is an important natural polymer for tissue engineering because of the protein's natural strength, biocompatibility, slow degradation rate, good water vapor and oxygen permeability, minimal inflammatory response and ability to be used in several forms [110]. Moreover, the resulting silk fibers are thermally stable up to 245 °C which ensures its stability at body temperature [120]. SF can be used as an electrospun scaffold, hydrogel, or film. Silk-based biomaterials can be tailored for vascular, bone or ligament applications.

### 2.5.1. Electrospinning Silk Fibroin

SF was first electrospun from HFP by Zarkoob *et al.* in 1998 and patented in 2000 [121,122]. Soon after, Sukigara *et al.* reported on the effects of the various electrospinning parameters on the morphology and fiber diameter of silk scaffolds. It was found that the SF concentration played a key role in producing uniform fibers [123,124]. In 2002, Jin *et al.* successfully electrospun SF from an aqueous solution by adding PEO to the silk solution in order to increase the viscosity [125]. Further studies, however, suggested that residual PEO in the SF scaffolds inhibited cell attachment and proliferation as well as adversely affecting the mechanical properties of the scaffold [126]. Regenerated SF electrospins as a random coil structure, but, as mentioned previously,  $\beta$ -sheet formations can be achieved by treating the scaffold with methanol or other cross-linking agent [127].

A rather unique property of electrospun SF is the ability to tailor its rate of degradation. While silk is considered to be non-degradable by most due to its retention of mechanical properties for periods longer than 60 days, it does in fact degrade. Silk will lose most of its tensile strength within a year *in vivo*, and will be unrecognizable at the implantation site within 2 years. Silk is considered biodegradable due to its vulnerability to bacterial and enzymatic degradation. Studies show that proteases will cleave the protein at the less-crystalline regions after which the resulting peptides can be

phagocytosed by cells [111,116]. Post electrospinning treatment of scaffolds with methanol can significantly decrease the scaffold rate of degradation [116,128]. Wang *et al.* showed that using an aqueous solution instead of an organic solvent like HFP can increase the degradation rate while promoting cell proliferation and penetration. SF scaffolds electrospun from an aqueous solution degraded between 2 and 6 months while those electrospun out of HFP lasted over a year *in vivo* in nude and Lewis rat subcutaneous implantation models. There is a wide variety of biocompatible polymers used for tissue applications besides SF. However, the degradation rates of other polymers cannot be tailored within such a high range as that of silk. Collagen degrades between 1 to 4 weeks and sometimes longer depending on the cross-linking process [129], whereas, PCL can last within the body for more than 2 years [130]. The synthetic polymer, PLGA (85:15) usually degrades within 26 weeks, but PLGA (50:50) will degrade between 6 and 8 weeks *in vitro* [131-133]. Silk scaffolds, however, can be modified to have similar degradation rates by changing the solvent for electrospinning [128].

SF scaffolds have proven a feasible option for vascular grafts with their unique mechanical properties and flexibility. SF produces tubular grafts that are porous and exhibit a high tensile strength which would be suitable for vascular applications [134]. Furthermore, tubular silk scaffolds electrospun out of formic acid can resist up to 575 mmHg in burst strength tests, which is more than four times the upper physiological pressure of 120 mmHg and twice that of pathological upper pressures of 180–220 mmHg [135]. Studies have shown that electrospun aqueous SF scaffolds promote aortic EC and arterial SMC growth and proliferation while withstanding vascular pulsating pressures [136,137].

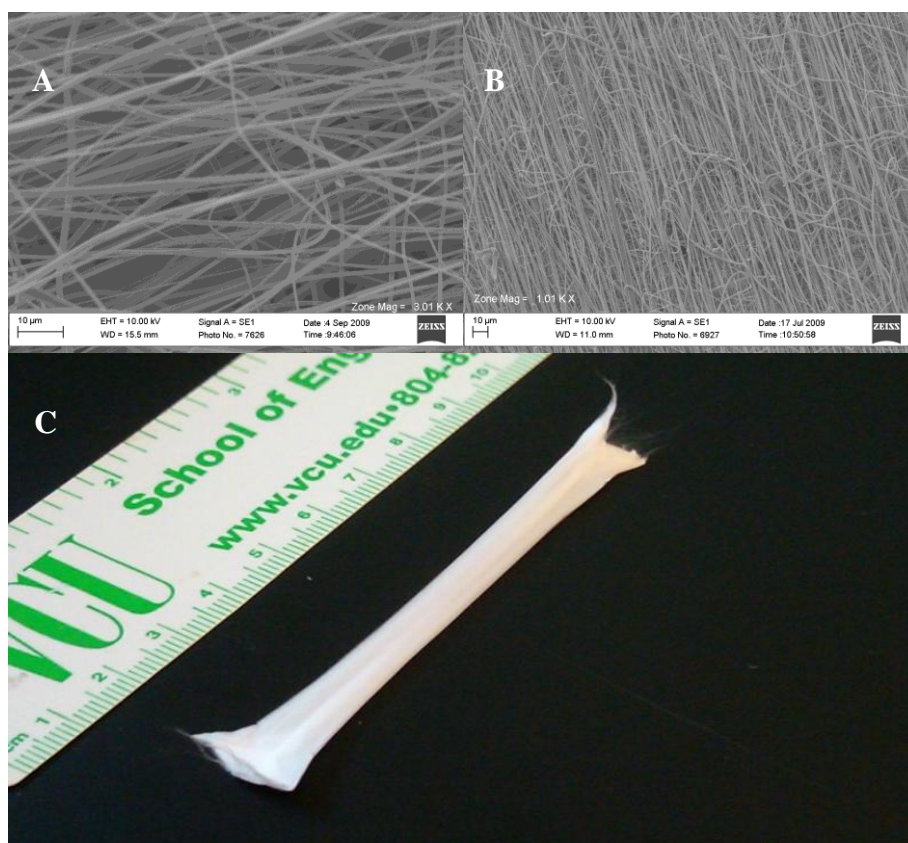
The ability to electrospin SF out of water provides a way to introduce growth factors or other components into the scaffold. In this way, SF scaffolds are a potential polymer for bone tissue engineering. When bone morphogenetic protein-2 (BMP-2) or nanoHA are incorporated into the electrospinning solution, the *in vitro* bone formation from mesenchymal stem cells greatly increased [138]. Such proteins and nanocrystals may not withstand the electrospinning process if the scaffolds were electrospun from organic solvents. As previously described, PEO may be added to the electrospinning aqueous solution to increase viscosity and aid in the electrospinning process. Studies have shown that while electrospun SF scaffolds support bone marrow stromal cell attachment and proliferation, residual PEO may initially inhibit cell attachment. However, after a few days in growth media, PEO will dissolve out from the scaffolds and cell attachment and growth will commence [126].

Ligament tissue engineering can also benefit from using silk-based scaffolds. An ideal scaffold for ligament tissue engineering must be biodegradable, porous, mechanically strong, and promote the formation of ligament tissue. Silk-based scaffolds meet all of these requirements. Studies show that anterior cruciate ligament (ACL) FBs and mesenchymal stem cells grow well on silk scaffolds as well as combined knitted silk scaffolds and silk sponges for ligament tissue engineering [108,139,140]. While silk's versatility as a biomaterial and its controllable mechanical properties make it a strong candidate for ligament and tendon tissue engineering, much of its usage in this area has been in the form of woven or knitted constructs, with little literature on creating an electrospun SF ligament scaffold.

The authors of this review have used the airgap electrospinning process to create highly porous structures of SF and SF blended with a number of synthetic polymers for ligament engineering [19]. These constructs exhibited fiber alignment, increased porosities, and improved cellular penetration

when compared to traditionally electrospun structures. In addition, using an in process fiber winding technique with the novel airgap electrospinner in Figure 2, it was possible to create electrospun structures with a wound center core and a highly porous outer layer (Figure 9). These structures were created by electrospinning a small volume onto the collecting electrodes rotating synchronously, followed by a duration of fiber collection on the electrodes while rotating in opposing directions to create a wound core structure, and finally another period of fiber collection with the electrodes rotating in the same direction to create a porous outer structure. These scaffolds maintained the high porosities required to promote cellular penetration while producing uniaxial tensile properties similar to those of the native ACL. Airgap electrospun structures composed of a 50:50 by volume blend of SF and PCL (SPCL) resulted in an average modulus of 144 MPa and a peak stress of 21 MPa; native ACL exhibits an average modulus of 113 MPa and a peak stress of 24 MPa.

**Figure 9.** SEMs of SPCL airgap electrospun structures demonstrating the high porosity outer shell (A, image at 3000 $\times$ ), the tightly wound inner core (B, image at 1000 $\times$ ), and a digital photograph of the multilayered structure (C). SEM scale bars = 10  $\mu$ m.



### 3. Current Challenges and Future Directions

While the electrospinning process has been used for nearly a century, the use of electrospinning to create an ECM analogue from natural polymers is still in its infancy. As such, there are a number of challenges that have yet to be overcome. A significant concern in the creation of ECM analogue structures is promoting sufficient cellular penetration and scaffold remodeling. If cells are not able to penetrate into the electrospun structure, simply choosing to proliferate and produce new ECM on the

surface, then the electrospun structure will be of little use as a tissue engineering scaffold. Ideally, the scaffold should become uniformly cellularized to promote adequate remodeling and eventual replacement with native matrix. While scaffolds made from naturally occurring polymers tend to fare better than their synthetic polymer counterparts when it comes to cellular penetration, they are still not perfect. While the incorporation of recombinant human growth factors to induce cell chemotaxis into an electrospun scaffold or the microintegration technique described previously can significantly enhance scaffold cellularity, both require further optimization and research to become common practice.

Another challenge in the use of electrospinning to create ECM analogues is the ability for the process to create truly three-dimensional structures that can replicate macroscopically the architecture of specific native tissues. While the microscopic architecture of an electrospun scaffold can be made to closely resemble the fibrous nature of the native ECM, the process has traditionally been limited to the creation of thin sheets and tubes. On the other hand, a process such as 3-D printing can create intricate three-dimensional structures, but lacks the precision to create fine nanofibers mimicking those of the native ECM. Future work in the field of electrospinning for tissue engineering will have to address and overcome this issue to be successful. The authors believe that the flexibility of the electrospinning process may be its greatest asset. As the previously described airgap electrospinning process, and previous research in the same vein have demonstrated, simply altering the way that fibers are collected can have a profound impact on the macroscopic architecture of an electrospun scaffold. Again, more research in this direction is needed, but the preliminary results have been promising.

As the electrospinning of natural polymers for tissue engineering applications continues to evolve, so too will our ability to control the process and tailor its output to our tissue specific needs. Having already harnessed the ability to create nanofibers nearly identical to those seen natively in the ECM, from the same molecules as the native ECM, it is only a matter of time before truly biomimicking scaffolds are created from electrospun natural polymers.

#### 4. Conclusions

While many obstacles remain in the creation of the ideal ECM analogue structure, the capability to electrospin the natural polymers found so prevalently in the native architecture has yielded promising results. The inherent bioactivity of these proteins, combined with the nanoscale fiber producing potential of the electrospinning process, has proven conducive to cellular adhesion, proliferation, migration, and differentiation. The mechanical properties of these electrospun biomimicking scaffolds, at times supplemented by synthetic polymers, have demonstrated mechanical properties capable of replicating those of an array of native tissues. Additionally, preliminary *in vivo* studies with electrospun natural polymer structures have proven the scaffolds to be adequately remodeled and integrated with native tissue. With the diversity of the native ECM, it is highly unlikely that a single processing technique or material will come to the forefront as the tissue engineering scaffold of choice, however the adaptability of the electrospinning process and its ability to create nanoscale fibers from the natural polymers found so prevalently in the native architecture holds great potential.

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