



Nanofibrous composites for tissue engineering applications

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Development of artificial matrices for tissue engineering is a crucial area of research in the field of regenerative medicine. Successful tissue scaffolds, in analogy with the natural mammalian extracellular matrix (ECM), are multi-component, fibrous, and on the nanoscale. In addition, to this key morphology, artificial scaffolds must have mechanical, chemical, surface, and electrical properties that match the ECM or basement membrane of the specific tissue desired. In particular, these material properties may vary significantly for the four primary tissues in the body: nerve, muscle, epithelial, and connective. In order to address this complex array of attributes with a polymeric material, a nanocomposite approach, employing a blend of materials, addition of a particle to enhance particular properties, or a surface treatment, is likely to be required. In this review, we examine nanocomposite approaches to address these diverse needs as a function of tissue type. The review is intended as a bridge between material scientists and biomedical researchers to give basic background information on tissue biology to the former, and on material processing approaches to the latter, in a general manner, and specifically review fibrous nanocomposite materials that have previously been used for cell studies, either *in vivo* or *in vitro*. © 2009 John Wiley & Sons, Inc. *WIREs Nanomed Nanobiotechnol* 2009 1 369–390

INTRODUCTION

Scope and Organization

Recent technical advances in regenerative medicine have necessitated the integration of materials science and the biological sciences. For instance, one primary focus of regenerative medicine has been the implementation of functional tissue engineering strategies to replace damaged or diseased organs, specifically with respect to the persisting problem of organ donor shortages, requiring the development of novel biomaterials to be used in conjunction with cell therapies. Much research has focused on creating scaffolds to support cells as they grow; such artificial supports have become more complex with technical developments, transforming from simple polymer

films to fibrous matrices with decreasing fiber-size-scale and increasing porosity, designed to closely resemble the natural extracellular matrix (ECM), which supports tissue growth and repair *in vivo*.^{1–5}

The purpose of this review is to bridge these two disciplines, providing information for material scientists on properties (mechanical, surface, chemical delivery, and electrical) that are beneficial in stimulating cells to create functional tissues; and familiarizing the clinical medical researcher with material science strategies to form nanofibrous composites with multifunctional properties, such as those listed above. The formation of a synthetic framework with appropriate surface properties onto which cells or tissues can engraft and which provides the correct sequence of mechanical, chemical, and electrical cues to develop working, functional units of tissue is the focus of this review. From a materials perspective, such a multifunctional array of properties is unlikely to be obtained in pure material; thus in this review, we focus on composite systems, including, design of co-polymers, polymer blending, incorporating particle into a matrix, surface modification, incorporation of protein aggregates

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and other chemical entities into a polymer matrix, and many other approaches. Because the ultimate goal of such research is to obtain the correct combination of physical and chemical stimuli to recreate the microenvironment cells experience *in vivo*, we will focus on nanofibrous systems, which have similar morphology to the natural mammalian ECM. Because each of the four mammalian tissue types (epithelial, nerve, muscle, and connective) requires different surface, chemical, mechanical, and electrical properties for an effective tissue scaffold support, we will divide our discussion by tissue type, with subdivisions focusing on the four particular material properties. Finally, we have chosen to focus on those works which have combined materials and biological research, that is, which have specifically tested cell response to a particular nanocomposite fibrous matrix to determine if the materials design is effective in controlling tissue development.

Many recent reviews on artificial tissue scaffolds for functional tissue engineering, with differing focus, have appeared. These reviews have highlighted current and past artificial tissue scaffold approaches, discussing the materials in use, including natural and synthetic materials^{4,6}; the methods of scaffold production, highlighting processing parameters,^{1,3,5,7–12} and overall future insight into this field.^{7,13}

Within the last decade, electrospinning has been widely used to fabricate nanofibrous scaffolds with high surface area (for cell adhesion and growth) and high porosity (for nutrient transfer).^{1,7,8,12,14,15} Currently, there are many excellent reviews related to fabrication and processing of nanofibrous mats for tissue scaffolds.^{12,16,17} In these works, the emphasis is on different strategies to produce tissue scaffolds and, primarily, how to control the electrospinning process to obtain desired scaffold morphologies.

We conclude this section with (1) a summary of scaffold properties generally identified to be beneficial for cell growth (primarily for our material science readers) and (2) an overview of the techniques utilized to form nanofibrous arrays, which may be of particular interest to medical and biological scientists. Similarly in the main document, we will first discuss what is known about mammalian tissue from a materials perspective (Section on Mammalian Tissue Overview), then address the materials used commonly in biomedical composites and their associated properties (Section on Materials for Artificial Scaffolds), before beginning our detailed review (Section on Nanofibrous Composite Scaffold Materials for Tissue Engineering). Finally, we conclude (Section on Conclusion) and offer interesting future directions and goals.

Materials Considerations in Cell Growth

Traditional culture systems have evaluated cell behavior in two-dimensional geometries by growing cells on flat surfaces and applying dosages of growth factors, cytokines, and other soluble factors to extrapolate their significance *in vivo*. Though this technique has long been used as a model system, investigators have become increasingly interested in three-dimensional (3D) cultures to produce functional tissue constructs that are able to truly mimic the *in vivo* environment.^{18,19} This 3D arrangement is important because cells and tissues require 3D spacing and positioning for their surface receptors to be able to evaluate the environmental cues surrounding them.^{19,20}

In order to perform appropriately, the key criteria for the scaffold include: specific ECM-like morphology, necessary mechanical properties, biocompatibility, support of normal cell processes (including adhesion, proliferation, migration, organization, and differentiation), and degradation (at a comparable rate to tissue formation).²¹ Research in this field has evolved from constructing inert structures to functionalized materials with enhanced physical and chemical properties that not only mimic the native morphology and chemistry of the ECM but also the series of signals required to evoke certain cell responses. When considering the overall composition and morphology of the ECM, three key characteristics are prevalent regardless of tissue type. They are as follows:

1. The ECM is composed of a heterogeneous combination of macromolecules including proteins and polysaccharides and inorganic matter (connective tissue only);
2. These ECM macromolecules are typically in fiber form which means that they possess a length/diameter ratio greater than 100;
3. The ECM macromolecules possess fiber diameters that are on the nanoscale, i.e. less than 500 nm.

As a result of these characteristics, nanofibrous composite non-woven mats are an ideal choice for tissue engineering because of their heterogeneous nature, matching that of the ECM, large surface area to volume ratio, ability to facilitate diffusion (as a result of high porosity), and tunability of physical properties.²² In addition, nanofibrous composite mats provide a compliant mesh that promotes *in vivo* cell phenotypes and overall tissue morphogenesis.²² As mentioned earlier, a synthetic ECM should not only meet the morphological similarities to the ECM but also the physical and chemical properties that

are required to guide tissue development and overall homeostasis.

Material Science Approaches to Form Nanofibrous Arrays

Current technologies that allow the fabrication of nanofibrous composite scaffolding are limited to three main processes: phase separation, self-assembly, and electrospinning.^{11,22,23} Phase separation is a technique that is based on the principle of a two-phase liquid–liquid phase separation to produce materials with nanoscale features. The morphology of these materials is similar to a foam with nanoscale walls, instead of fibers, creating a porous shell.^{24,25} Self-assembly is the spontaneous organization of molecules that form ordered structures on the nanoscale with fiber diameters ranging from 1 to 10 nm.^{26,27} However, self-assembly is complicated, time-consuming, and highly limited to protein-related materials which have specific molecular interactions. Electrospinning is the most common technique because of its relatively low cost, versatility, and overall simplicity. Multiple reviews have been published on the electrospinning of polymeric nanofibers and we refer the reader to these for the intricate details about electrospinning.^{1,12} The main drawback of electrospun mats, however, in addition to the slow fabrication rates and limited 3D nature, is the lack of strength and functionality where most tensile strength and modulus data have been reported to be in the range of kPa to a few MPa.^{28–30} Therefore, this review focuses on research utilizing multi-component nanofibrous materials to impart particular functionality to the scaffold, whether it be for strength enhancement, morphology optimization (available surface area/porosity), or improved cell function (adhesion/growth/differentiation).

MAMMALIAN TISSUE OVERVIEW

Composition of the ECM

The ECM is composed of a meshwork of macromolecules that provide biochemical and biophysical cues for cell function. Through different combinations, spatial organizations, and biochemical interactions of these macromolecules, the different tissues of the body are formed. Natural ECMs are composed of two types of molecules: fibrous proteins and polysaccharides.^{2,31} Proteins can loosely be defined by the presence of a peptide bond and predominately include collagens (up to 28 varieties), laminins, fibronectins, and elastins. These proteins possess four states of organization: primary, secondary, tertiary, and quarternary. For

fibrous proteins, their secondary state largely dictates the resulting properties and can be divided into four secondary conformations: random coil, collagen triple helix, β -sheet, and α -helix.³² Polysaccharides of the ECM are known as glycosaminoglycans (GAGs), and are unbranched polysaccharide chains composed of repeating disaccharide units including hyaluronan (nonsulfated), keratin sulfate, chondroitin sulfate, and heparin sulfate.² GAGs can be characterized by being negatively charged and thus are able to hold large amounts of water because of their polar nature.

The nanometer-scale structure of the ECM provides a fibrous web to support cells and a semi-rigid structure to guide their behavior. The native ECM components are organized into fibrils ranging from tens of nanometers to micrometers in scale.³² The fibrous structure allows for cell guidance and provides a hydrated porous network that allows for chemical interaction with the surrounding environment. In addition, these matrix fibrils serve as chemical reservoirs for the storage of bioactive factors for the regulation of cell migration, proliferation, and differentiation.³³ These reservoirs include growth factors, trace elements, and secreted proteins known as cytokines.³³ In general, cells are able to attach and deform the substratum via linkages known as integrins. These cell–ECM contact points allow interactions where receptor-mediated signaling can occur via mechanical deformation through cytoskeletal elements, including actin.³⁴ This phenomenon has been termed mechanosensing, and the ECM has been shown to control cell fate as a function of the physical properties, including stiffness and conductivity to distinguish a few.³⁴

Overview of Tissue Types

Tissues can be defined as an assembly of cells surrounded by an ECM. In vertebrates the main tissue types are nerve, muscle, epithelial, and connective tissues.^{35,36} Each of these tissues is held together by a supportive network of secreted extracellular macromolecules; however, the composition and physical features of this matrix greatly vary based on the tissue type and its physiological properties. As discussed above, in general, the ECM is composed of a variety of proteins and polysaccharides that assemble into an organized meshwork leading to cell function and overall homeostasis.³² Particular ECM composition determines how the cells will interact within this environment by allowing specific cell–cell or cell–matrix interactions to occur.

Nervous

Nervous tissue is mainly comprised of neurons and supporting glial cells, which allow the transfer of chemical and electrical signals to the neurons to mediate a response. This type of tissue allows the movement of ionic and electrical gradients by a mechanism known as an action potential that terminates as a synapse. Electrical gradients are transmitted through the cell and then pass along the cell body where they are transmitted to another cell at what is known as a synaptic junction.³² This junction is where a large amount of information (including, amplitude, frequency, and rate of the signal propagation from the stimulus at the initiating neuron) is transferred. Nervous tissue is well insulated and responds to stimuli by the production of electrical activity via the movement of ions in and out of the neuronal cell body and down the axon to form an action potential. Scaffold materials for this tissue permit changes in cell morphology of the cell body with extended dendrites, and provide necessary insulation to prevent dissolution of ionic currents. Nervous tissue ECM is primarily composed of laminins for the insulation and passage of electrical gradients.³⁷ During remyelination or the regeneration of the insulating sheath along the axon, laminins have been hypothesized to induce cell proliferation, and separation of axons.³⁷

Muscle

Muscle is tissue that is specialized for contraction and has three main derivatives: skeletal, cardiac, and smooth. Muscle tissue is arranged in a striated manner which is necessary for adhesion and to allow contraction to occur between the different spindle fibers of the tissue. Skeletal muscle is recognized by its striated manner and is the prime mover for voluntary actions. Cardiac muscle is a tissue that is electrically coupled via gap junctions, interconnecting ion channels between cells, to allow for a succinct contraction–relaxation mechanism. Recent research has assessed some of the physical properties of cardiac tissue by immunochemical staining of collagens, laminins, fibronectins, elastins, and chondroitin sulfates.³⁸ As a result of the high cellularity of this tissue, the ECM is mainly comprised of a low amount of collagen used for the ordering of cells in bundles and fascicles.³⁹

Epithelial

Epithelial tissue is primarily used to form membranes from the inner to outer surfaces of the human body, including the skin (the largest organ of the human body based on surface area and depth) to

the lining of the organs of the gastro-intestinal tract. Epithelial tissue is highly cellular in the profile of cell sheets with limited ECM material, and typically displays a stratified morphology with multiple cell–cell connections known as desmosomes and connections to the ECM.³² Depending on the organ's primary function, epithelial membranes can allow either minimal or maximal interaction (i.e., absorption) with their surrounding environment. A key feature of epithelial tissues is stratification which is achieved by the presence of the basement membrane. The basement membrane forms a connection site with epithelial cells via hemidesmosomes. Microscopic analysis has revealed that the basement membranes possess mean fiber diameters of 52 ± 28 nm and pore diameters of 82 ± 49 nm.³⁹ In addition, the main components of this membrane are laminins, Type IV collagen, nidogen, and perlecan.³¹ These components are notable in the ECM of epithelial tissues because they promote specific functions including differentiation and cell migration. The size-scale and ECM components provide a rational basis for design of synthetic ECMs for epithelial tissues and are a testament to the diversity of ECM characteristics.

Connective

Connective tissue provides structural and mechanical support for the body and works in conjunction with muscle tissue for coordinated and reflexive movements by acting as the lever arm. Connective tissue can be grouped into two main groups: mineralized and non-mineralized tissues. Bone and teeth are largely comprised of Type I collagen and hydroxyapatite (HA) crystals. Articular cartilage is primarily composed of collagen Type II and some Type IV. Cartilage is unique in that the orientation of fibrils change based on the location within the tissue from being perpendicular to the surface of bone, to randomly oriented fibrils. Ligaments and tendons are mainly composed of Type I collagen (<70%), elastins, and a low amount of proteoglycans.

Regarding the four classes of tissues in the body, their properties vary widely, based upon the arrangement and contribution of the ECM and the specific cell types for that organ. Past research noted that by simply changing the elasticity of the synthetic ECM, stem cells can be directed down specific lineages.⁴⁰

MATERIALS FOR ARTIFICIAL SCAFFOLDS

This section focuses on specific materials used in multi-component nanofibrous scaffolds. The two main

classifications of materials are matrix materials (used to fabricate the fiber structure itself) and the functional material (which can either be a reinforcing particle or a surface coating). The general matrix materials will be discussed here, whereas many examples of functional materials appear in the Section on Nanofibrous Composite Scaffold Materials for Tissue Engineering, categorized by specific properties being targeted (that is, mechanical, surface, chemical, or electrical).

Researchers take two main approaches, bioresorbable polymers or permanent, non-biodegradable materials, to choose the materials for a fibrous tissue scaffold. A comprehensive review on materials used for tissue and organ regeneration was published by Panitch and coworkers⁴¹ and discusses materials not limited to those used in nanofiber fabrication. The strong focus on bioresorbable scaffolds is because of the short-term nature of the function of the scaffold (which will be replaced by a natural ECM secreted by the cells), and the desire for it to degrade such that no foreign materials remain. In addition, many bioresorbable polymers are approved by Food and Drug Administration (FDA). Alternatively, a non-bioresorbable scaffold may be of interest if, for instance, the scaffold is filled with a material (such as a nanoparticle) that may have toxicological concerns. In that case, permanent encapsulation ensures that the functional particle will remain trapped, and therefore 'invisible' to the body.

Boland et al.¹⁴ have written a comprehensive review focusing on natural and artificial bioresorbable polymers such as collagen, poly(lactic acid) (PLA), poly(glycolic acid) Poly(Glycolic Acid) (PGA), and poly(caprolactone) Poly(Caprolactone) (PCL). The copolymer poly(L-lactide-co-glycolide) Poly(L-Lactide-Co-Glycolide) (PLGA) is also commonly used. In Boland's work, the idea is to fabricate a scaffold for cell seeding and growth with the intent that over time, the scaffold will resorb/erode into the body with that volume replaced by tissue. Strategies for biodegradable materials have focused on using blends of different polymers (specifically PLA and PGA) as well as co-polymers of polymers such as PLA and PGA to control the rate of erosion. Li et al.⁴² have evaluated combinations of blends and co-polymers of poly(alpha-hydroxy ester) based polymers for optimal mechanical and biological responses. Poly(propylene fumarate),⁴³ gelatin,²³ chitosan,⁴⁴ and peptide-amphiphile^{45,46} have received attention as fibrous scaffolds in multi-component systems, as well as some less common materials such as polyelectrolyte complexes,⁴⁷ and poly(3-hydroxybutyrate-co-3-hydroxyvalerate).^{48,49}

Polyelectrolyte complexes are composed of both cationic materials (chitosan, poly-L-lysine for instance) and anionic materials (hyaluronan, alginate, and heparin). Chitosan, hyaluronan, heparin, and alginate are all linear polysaccharides and have been used in the production of nanocomposite scaffolds because of their source availability and biofunctionality, meaning that they offer low inflammation reactions and are biocompatible. Hyaluronan, also known as hyaluronic acid, is an anionic, nonsulfated, high molecular weight GAG. Chitosan, the deacetylated derivative of chitin, is similar in structure to GAGs within the mammalian ECM. Heparin is the most negatively charged polysaccharide as a result of having multiple sulfate groups along its backbone. Alginates are linear polysaccharides, derived from seaweed, and are composed of D-mannuronic and L-guluronic acid residues. When in the presence of divalent cations, notably calcium, a semisolid gel can be formed through the ionic crosslinking between the carboxylic acid groups located along the polymer chain. Other materials that form polyelectrolyte complexes include poly-L-lysine which displays an amino group along a linear hydrocarbon backbone.

Non-bioresorbable scaffolds, while less common, have received some attention in multi-component scaffolds as well. Polycarbonate urethane with carbon fiber⁵⁰ and silk/poly(ethylene oxide) (PEO)⁵¹ has been evaluated for bone cell adhesion.

NANOFIBROUS COMPOSITE SCAFFOLD MATERIALS FOR TISSUE ENGINEERING

General Principles

Morphology

There is compelling evidence that fibrous scaffold morphologies are advantageous to porous foam/gel morphologies as recently shown by Alvarez-Barreto et al.⁵² Therefore, there has been a much research in the last decade devoted to the development of fibrous scaffold substrates. Controlling the fibrous scaffold morphology [size and volume fraction of pores as well as the fiber diameter (and therefore the surface area available for cell adhesion and growth)] and orientation is of considerable importance to a tissue engineer.^{53–55} For instance, to some degree, this can be achieved by control of the processing conditions in electrospinning as well as the solution conditions (such as the polymer concentration, the solvent system, and additives to change the net charge density of the solution) which can in turn control

the size of the fibers produced.^{1,7,8,12,14,15,56,57} The effect of morphology on cells was demonstrated by Pham et al. where the authors measured cell infiltration based on fiber size and resulting pore size.⁵³ Larger fibers were able to generate scaffolds with considerably larger pores altering the ability of cells to migrate within the 3D matrix. In addition to controlling processing conditions, the removal of sacrificial fibers can increase cell migration and penetration providing an enhanced porosity; even more site-specific techniques have shown the ability to structure nanofiber scaffolds via laser ablation.⁵⁵ However, there are limitations to minimum achievable fiber diameter in all three primary nanofiber-forming techniques (phase separation, self-assembly, and electrospinning), and therefore novel approaches to obtain fibrous mats are of interest. Researchers have also evaluated how the orientation of fibrous mats (compared with the typical random morphology) can affect guided growth of cells on the scaffold.¹⁶ When addressing how the ECM morphology for mineralized connective tissue is arranged, researchers have attempted to mimic its high porosity by having macropores of $>50\ \mu\text{m}$, to allow for cell migration and adequate transport of nutrients and wastes.⁵⁸

Mechanical

The mechanical properties of the scaffold are also an important part of substrate design,⁵⁹ specifically in the formation of structural tissue, such as bone.^{23,43,50,60–69} Originally, it was hypothesized that scaffold should possess similar mechanical properties to the actual tissue solely for structural purposes (compressive stiffness for bone and elasticity for skin).^{13,17,70–72} Recently, however, researchers have discovered that the stiffness of the scaffold has an important effect on the cell differentiation process.^{34,73} These findings place an even greater importance on the mechanical properties of tissue scaffolds to not only support the appropriate loads and deformations but also to actively assist in the differentiation of cells down specific lineages.

The tissue engineering field has had a strong focus on bone and cartilage tissue^{23,43,50,60–69} with developing high stiffness scaffolds as a significant priority. Many strategies have focused on the incorporation of rigid particles (such as HA, calcium carbonate, and beta-tricalcium phosphate) into the fibrous matrix to improve strength and stiffness.^{17,43,62,64,70,74,75} For instance, research has determined that loading levels of up to 75 wt%, these composites typically exhibit significant increases in elastic modulus and compressive strength compared with neat, porous, biodegradable

polymer systems.⁷⁶ However, the increased properties of these porous composite systems are still not equivalent to those of cortical or cancellous bone.⁷⁶ In addition, the use of blends and co-polymers, typically PLA and PGA based (including those utilizing materials such as gelatin, collagen, and chitosan), has also been used to optimize stiffness.^{23,44,61,65,77,78} For instance, PLA and PGA blending has been seen to increase the Young's Modulus and overall long-term sustainability in culture with bone-derived cells and mesenchymal stem cells (MSCs).^{46,65} Less common have been approaches using non-bioresorbable materials such as carbon fibers⁵⁰ and carbon nanotubes.⁵⁷ In both cases, the ultimate goal was not only to increase scaffold modulus, but also to increase cell adhesion and growth via changes in morphology as well. However, additive toxicity, specifically nanoparticle toxicity, is a significant concern and is currently being addressed by several research groups.^{79–81}

Mechanical properties of natural biopolymers are generally enhanced by addition of synthetic materials. Single-component fibers of proteins from the ECM show poor mechanical properties.⁸² Kwon's study on poly(lactide-co- ϵ -caprolactone) (PLLC) blends with collagen showed a decreasing tensile strength with increasing collagen content.⁸³ For PLGA–gelatin–elastin fibrous scaffolds, mechanical measurements showed increases in modulus and strain to failure with increasing PLGA.⁶¹ One goal of this work was to obtain non-dissolving natural polymer blends (without crosslinking) by electrospinning a single homogeneous solution of the three components. Fibrous scaffolds exhibited swelling and subsequent permanent changes when exposed to aqueous media, but did not dissolve. In this case, blend elasticity was enhanced with hydration,⁶¹ a result which emphasizes the need to measure scaffold properties in the physiologically relevant environment in which they will be utilized, particularly when natural biopolymers (which are sensitive to water content) are used. Polyaniline (PANi) blended with gelatin and then electrospun provided both enhanced conductivity (discussed below) and increased tensile strength and modulus.⁸⁴ Not surprisingly, elasticity decreased with increased PANi content, dropping from 96 to 9% from pure gelatin to 45:55 PANi:gelatin volume ratio,⁸⁴ highlighting the known reality that simultaneous strength and toughness are difficult to obtain and thus ideal mechanical properties (i.e., the relative importance of modulus vs. elasticity) for particular tissues need to be identified and obtained via composite design.

Mechanical properties can be modified by polymer blending or surface modification designed to

achieve other aims. In particular, as discussed above, blending with natural biopolymers (such as chitosan) or water-soluble materials [such as PEO or poly(vinyl alcohol) (PVA)] may result in uncontrolled swelling or formation of porous structures in an aqueous environment with subsequent morphological and mechanical changes. Bhattarai et al. fabricated nanofibrous scaffolds containing PEO and chitosan⁴⁴ by electrospinning, with chitosan serving to support cell attachment, differentiation, and growth. By limiting the chitosan fraction and use of a surfactant, these authors produced a fibrous morphology stable in immersion for 4 weeks in water.⁴⁴ Obviously, mechanical properties will also change with scaffold degradation. Duan studied the mechanical properties of mixed scaffolds of PLGA and chitosan/PVA fibers, crosslinked with glutaraldehyde vapor, during degradation^{71,72} and found that elongation showed the greatest sensitivity, dropping dramatically after 1 week (from 15 to 5%), while strength decreased by a factor of two, and modulus by about 30%. With respect to surface modification, Zhu et al. utilized aminolysis to surface modify PLLC fibers with fibronectin.⁸⁵ Such breaking of the surface polymer chains made the scaffold more brittle, but it maintained similar tensile strength and the scaffolds still had greater elasticity than neat natural biopolymers (such as collagen)⁸² or neat PLA, indicating that co-polymer formation (even with subsequent surface modification) was a useful approach.⁸⁵ Park et al. saw elongation values of >100% for PGA, PLGA, and poly(L-lactic acid) (PLLA) mats with acrylic-acid grafting (after plasma treatment), although no comparison to untreated materials is provided.⁶⁸

Surface Modifications

In addition to the efforts focused on optimizing scaffold morphology and mechanical properties, significant efforts have focused on surface modification of fibers and scaffolds to promote cell adhesion, growth, and differentiation^{56,68} and in many cases selective cell adhesion and growth.⁵⁰

Plasma treatment with *in situ* grafting of acrylic acid⁶⁸ has been shown to improve fibroblast proliferation. Similar strategies to plasma treat and graft acrylic acid have also been used on HA particles to create novel scaffolds for improved cell function,⁸⁶ although not in a nanofibrous geometry. Blending HA with PLA has shown binding between the COOH in PLA and the Ca²⁺ of HA leading to coarser fibers and better cell adhesion and proliferation.⁷⁵ Changes in surface energy of carbon fibers have been shown to selectively affect adhesion of different cell types.⁵⁰ Other surface treatment approaches have successfully coated proteins on nanofibrous surfaces for improved

tissue formation⁸⁷ and used peptide–amphiphile molecules with the sequence of arginine–glycine–aspartic acid to improve osteogenic differentiation.^{45,46} Many researchers have also reported improved cell adhesion by blending polymers and/or forming a composite fibrous structure^{23,43,44,48,63,64,69,74,77,88–90} using materials such as HA, tricalcium phosphate, or chitin nanoparticles. Yim et al. used human MSCs (hMSCs) encapsulated in (and seeded on) polyelectrolyte complexation fibers to improve proliferation and differentiation.⁴⁷

Carbon nanotubes and nanofibers have several properties that may be of value in the development of novel devices for bone reconstruction, including increasing cell adhesion. The aspect ratio and physical shape of these fibers mimic the crystalline HA structures of natural bone. By altering dimensions of carbon fibers, one can increase area and subsequently increase adhesion of any cell line. In a study by Price et al., the surface energy was controlled by creating carbon fibers either with or without a pyrolytic polynuclear aromatic hydrocarbon (PAH) outer layer.⁵⁰ The surface energy was found to be about five times higher on those fibers without the pyrolytic layer than the ones with them. A carbon nanofiber formulation with high surface energy (PS fibers) and small diameter could be used to create a scaffold with enhanced adhesion properties. On the other hand, a material that would promote fibroblast, chondrocyte, or smooth muscle adhesion could be created by using carbon nanofibers with the PAH layer intact and with larger conventional diameters. These surface properties vary for different cell lines merely by the cell surface receptors available on the cell.

Although not performed on multifunctional nanofibrous scaffolds, research by Curran et al. has focused on a fundamental yet simplistic model to gauge the effect of surface chemistry on the lineage potential of MSCs.^{91,92} (Curran's past works focused on modifying substratum with different chemistries including carboxyl, hydroxyl, amino, silane, and methyl termination.) The results from this study revealed that by altering the surface chemistry, the MSCs exhibited changes in morphology, ECM production, and gene expression. In particular, the authors were able to distinguish between stem cell (methyl termination) osteoblastic (amino and silane termination) and chondrogenic (carboxyl and hydroxyl termination) phenotype expression which was material-driven with little sensitivity to media conditions.⁹² Specifically osteogenic surface chemistries yielded a higher expression of osteogenic markers including osteocalcin and collagen type I,

while chondrogenic surface chemistries downregulated osteogenic marker expression yet supported the expression of collagen type II, a positive marker for chondrogenesis. Although this work did not look at multifunctional substrates with more than one surface termination group, it raises a very important prospect for the development of tissue boundaries which can be formed by modifying the surface chemistry of the scaffold.

Chemical Delivery and Scaffold Degradation

Degradation rates of nanofibrous composites are of importance for all tissue types. Two biodegradable polymers that are commonly blended to tune degradation rates are PGA and PLA. PGA, owing to its hydrophilic nature and low crystallinity, is sensitive to its environment and tends to degrade in aqueous solution. With the addition of a methyl group, PLA is more hydrophobic and degrades more slowly than PGA. PLGA co-polymers are amorphous and exhibit fast degradation rates. During degradation one issue can be that inter-fiber pores close as a result of polymer swelling and the scaffold shrinks because of the fiber shortening. This decreases the porosity during degradation.⁶³ High porosity is essential as it provides more structural space for cell accommodation and makes the exchange of nutrient and metabolic waste between a scaffold and environment more efficient.⁶⁹

Zong et al. electrospun various compositions of poly(lactide-co-glycolide), PLLA, and the diblock copolymer poly(ethylene glycol)-poly(D,L-lactide) in order to tune scaffold degradation.⁹³ For instance, PLGA 10:90 blended with PLLA showed weight loss of 20% over 7 days in aqueous solution. The authors noted that such accelerated scaffold degradation had a negative impact on cell growth causing cell clustering and poor morphology. Duan^{71,72} fabricated mixed scaffolds composed of PLGA and chitosan-PVA fibers by simultaneous electrospinning from two separate syringes and collecting on a rotating drum. The fibers were crosslinked with glutaraldehyde vapor. The mixture of fibers attempted to mimic two primary components of the ECM, collagen (modeled by the more mechanically robust PLGA) and GAGs (modeled by the chitosan component). A blend solution with PVA enabled electrospinning of chitosan. PLGA was selected as a biodegradable component where decomposition rate can be tuned by altering the ratio of co-polymers. However, although very little decomposition was observed for pure PLGA scaffolds, inclusion of the chitosan fiber in the scaffold increased aqueous absorption⁷² and subsequently led to quicker degradation of the PLGA.⁷¹

Chemical delivery can occur via three general mechanisms. The first is delivery of the active species at the fiber surface via diffusion from core of the fiber to the surface. The second is the use of a surface mechanism to release the active species in a controlled manner. The third is the degradation of the scaffold surface which results in the surface release of the active species. Probably the most popular mechanism has been to control the degradation of the matrix to release the active species. For instance, one can blend different polymers (with different degradation rates, such as PLA and PGA), synthesize co-polymers from these repeat units, or produce layered structures (such as core-sheath fiber morphologies). Diffusion-mediated release has been reported in nanofibrous mats as well.⁹⁴ Core-sheath nanofibers⁹⁵ (with a protein/poly(ethylene glycol) water-soluble core) or phase-separated blends of biodegradable polymer with water-soluble polymers, such as PEO, have also been utilized for protein delivery to explicitly form protein-rich regions that slowly dissolve when placed in aqueous environment (see for instance Ref. ⁹⁶).

An unusual approach for chemical functionality is to encapsulate cells within the fiber or scaffold matrix during fabrication. In principle, encapsulated cells can provide a variety of chemical cues to cells subsequently seeded on the scaffold, or serve as the initial population for tissue formation.^{47,97} For instance, Yim et al. reported encapsulation of MSCs in macro-sized fibers.⁴⁷

Electrical

Electrical stimulation of cells in culture has been of interest, especially in the last 5 years.⁹⁸⁻¹⁰¹ Seal et al.⁴¹ have reviewed conductive materials used in tissue regeneration and highlight a study by Jakubiec et al.¹⁰² that suggests an optimal, intermediate level of conductivity for endothelial cell migration and viability on polypyrrole-coated polyester. To a much lesser degree, researchers have investigated the effects of electrical stimulation on cell growth using nanofibrous scaffolds.⁸⁴ Here, polyaniline was blended with gelatin and electrospun to form a nanofibrous composite. While the results were promising in terms of supporting cell attachment and growth, there was little difference in the final cell density and morphology as compared with the control tissue culture-treated plastic. This will continue to be a growing area of interest, especially as researchers develop new and innovative ways to fabricate conductive nanofibrous scaffolds.

NERVE TISSUE

Overview

Only a few publications have addressed interactions between nanofibrous composite materials and cells of the nervous system, either neurons or the supporting glial cells. A fundamental issue in nerve-based tissue engineering is repair of severed nerves where neuron growth between the two 'stumps' requires both a pathway, which guides cells to bridge the gap, and prevention of scar formation by non-neuron cells. A stable scaffold structure and sustained biochemical release may be particularly important as nerve re-growth *in vivo* is particularly slow. Yang et al. argued that glial scarring, which limits neuron migration (or axon growth) into damaged neurological tissue might be prevented by use of a scaffold with pore size less than 10 μm to block the migration of scar-forming cells to a damage site.¹⁰³ McKenzie found that carbon nanofibers decreased astrocyte (a scar-forming glial cell) adhesion and activity perhaps because of nanostructured roughness.¹⁰⁴ In addition, single-component aligned nanofibrous scaffolds have been utilized to study contact guidance of neural stem cells, with the hope of forming conduits to bridge gaps in peripheral nerves and thus facilitate repair.¹⁰⁵ Finally, conductive particles (particularly carbon nanotubes) have been utilized as nanoelectrodes to interface with neurons,^{104,106–109} and conducting polymers have recently been electrochemically polymerized within neural tissue.^{110,111} These reports indicate that nanofibrous composites designed for neurological tissue growth may be an expanding area in future years.

Surface Modification

Surface modification to enhance adhesion and particularly facilitate neurite outgrowth has been an area of focus for neuronal tissue engineering. Electrospun polyamide nanofibers were modified with peptides derived from human tenascin-C, an ECM glycoprotein and improved attachment and neurite generation and extension was observed.¹¹² Electrospun poly(L-lactide) nanofibers coated with laminin and basic fibroblast growth factor (bFGF) to encourage neurite extension were formed into random and aligned scaffolds and exposed to rat dorsal root ganglion.¹¹³ While alignment alone encouraged extension and minimized branching, the effect was enhanced by the surface factors. In fact, surface-bound bFGF was as effective as bFGF placed in solution, indicating that bioactivity was largely retained in this case.

Schnell used a collagen–PCL blend to facilitate cell adhesion, proliferation, and guidance on aligned scaffolds of electrospun fibers.¹¹⁴ Here a particular focus was cell-free implants where native cells colonize the implanted material. For single-cell studies, two types of glial cells (Schwann and olfactory ensheathing), fibroblasts, and neurons were separately tested, along with a transplanted tissue from chick dorsal root ganglia. In the single-cell study, neurite projections were longer on the collagen blend (in comparison with a pure PCL scaffold). Similarly, elongated Schwann or olfactory ensheathing cells (separate experiments), aligned along the fibers, were observed on the collagen blend substrate. In addition, Schwann cell migration from the explanted tissue was also enhanced. Mobility of Schwann-type cells may be crucial to form a supporting structure for axon growth. Fibroblast morphology (used as a general model cell type in this study) was the most sensitive to the presence of collagen with round cells in clusters occurring on the PCL in contrast to extended, aligned fibroblasts on the blend.¹¹⁴

Chemical Delivery and Scaffold Degradation

Use of a nanocomposite for chemical delivery (again to facilitate neurite outgrowth) has also been reported. Chew et al. reported nanofiber formation via electrospinning of a co-polymer of ϵ -caprolactone and ethyl ethylene phosphate with human nerve growth factor (NGF) stabilized with bovine serum albumin (BSA).^{115,116} Sustained release of growth factor is important as many biochemicals have short-lived bioactivity in solution. Here, the co-polymer blend was designed to increase the rate of polymer degradation in order to deliver the nerve growth protein. A model comparison system, using BSA conjugated to a fluorescent dye, revealed that the protein aggregated into particles throughout the fibers. This result is consistent with the solution-formation process where the aqueous (protein) and the organic (polymer) components were mixed and then vortexed to form an aqueous suspension within the organic electrospinning solution (see also Ref. ¹¹⁷). Particle formation by water-soluble proteins mixed into organic electrospinning solutions has also been observed by other workers.⁸³ After an initial spike of 20% protein release (from protein on the surface), a steady increase was observed to 80% release at approximately 2 months. The authors hypothesized protein release was as a result of diffusion out of the polymer matrix as no polymer degradation was observed. The extracted protein was tested for bioactivity with rat PC 12 cells, a clonal cell line from pheochromocytoma adrenal gland tumors which

responds to NGF by ceasing cell-multiplication and extending neurite-like processes.¹¹⁸ This experiment showed that some bioactivity was retained after the electrospinning process. (See also Ref. ⁹⁶.) An animal model study was also conducted.¹¹⁶

Tuning of mechanical and electrical properties for nerve tissue engineering has not yet been discussed within the confines of nanofibrous composites.

Muscle Tissue

Surface Properties

Many efforts toward muscle tissue engineering via fibrous nanocomposites have focused on modifying surface properties, while maintaining mechanical strength, as discussed in detail in the barrier (epithelial) tissue and general principles sections, respectively. Li et al. utilized electrospinning to create fibers of poly(lactic-co-glycolic acid)–gelatin–elastin blends to ensure proper surface chemistry, and retain mechanical properties without crosslinking.⁶¹ Cardiac myoblasts proliferated equally well on the PLGA fiber and blended fiber scaffolds; however, substrate-mediated differences in cell morphology were observed. In particular, cell penetration into the scaffold (as opposed to remaining on the surface) was observed for the PLGA–gelatin–elastin blend scaffolds, despite lower modulus than the PLGA scaffolds. Li et al. argued that although increased elasticity would seem to allow cell migration (see the mechanical section below) into the scaffold, the improved surface properties must have overcome this tendency.⁶¹ Cell migration of smooth muscle cells into collagen-coated PCL matrices was also observed by Venugopal et al. However, they note that cell proliferation and growth rate on collagen-coated PCL, although improved compared with a PCL substrate, was less than that on pure collagen fibers.¹¹⁹ Baker et al. electrospun polystyrene and modified the surface by plasma treatment [which is commonly used for tissue culture polystyrene (TCPS)], which lead to a two-fold increase in cell attachment for smooth muscle cells, as a result of improved hydrophilicity.¹²⁰ Zong found that primary cardiomyocytes had the highest cell density, the most natural morphology, and the highest electrical activity on the most hydrophobic surface (PLLA) for both aligned and random electrospun scaffolds,⁸³ compared with poly(lactide-co-glycolide) and the diblock co-polymer poly(ethylene glycol)-poly(D,L-lactide). This result highlights differences in cell response to surface properties as a function of cell type and perhaps particular processing protocols.

Kim and Park utilized an amino-terminated block co-polymer (PLGA/PLGA-b-PEG-NH₂) to tether an RGD cell-adhesive peptide (Gly-Arg-Gly-Asp-Tyr),¹²¹ where PEG is a poly(ethylene glycol) co-polymer. This peptide contains the RGD (Arg-Gly-Asp) sequence found in many ECM proteins (e.g., collagen, fibrinogen, and laminin) associated with adhesion. Comparing the hydrophilic, but not specifically bioactive, NH₂ surface with that after addition of RGD, the RGD-terminated surface had the greatest fibroblast cell adhesion (ability to bind cells from solution phase), indicating that specific biological triggers facilitated cell attachment. Comparing RGD-terminated PLGA and pure PLGA (not aminated), cells also had larger spreading rates, penetrated into the scaffold interior, and formed actin fibers, on the surface-modified scaffolds.¹²¹ A similar approach, not yet utilized for a nanofibrous composite, is coating scaffolds with peptide ligands, where the peptide sequence can be controlled to tune surface changes and trigger particular cellular responses.¹²²

Chemical Delivery and Scaffold Degradation

Stankus integrated smooth muscle cells into scaffolds by simultaneously electrospaying cells while electrospinning poly(ester urethane)urea on the same target, with the goal of maximizing cell seeding throughout the matrix (100–500 μm in thickness).⁹⁷ Cells retained bioactivity after electrospaying, in contrast with attempts to physically spray cells in solution for incorporation. Viable cells were found throughout the scaffold volume via this technique; however, for thicker scaffolds, growth was limited under standard static culture conditions, which the authors hypothesized did not allow effective exchange of water and nutrients to the interior of the matrix. Cell proliferation was improved by utilizing a perfusion bioreactor which utilized fluid flow to ensure exchange of media with the interior of the matrix.⁹⁷

Mechanical Properties

Mechanical properties of muscle tissue are generally anisotropic. A related issue for muscle cells is de-differentiation in homogeneous (no strain or alignment) environments. Cells which are in functional phenotypes and aligned with respect to the ultimate strength axis of the muscle will secrete collagen, elastin, actin, and myosin in an anisotropic distribution, which is needed to obtain the correct final mechanical properties of the tissue. Many workers^{123,124} have observed secretion of collagen and elastin by smooth muscle cells in random scaffolds (indicating alignment because of contact guidance). Several groups have evaluated the efficacy of aligned

scaffolds (in addition to contact guidance) to direct cell growth. Tuan's group studied aligned scaffolds as a means to provide anisotropy, and found changes in human MSC morphology with more elongated cells and actin fibers formed along the alignment direction.¹²⁵ Similarly Baker et al. studied smooth muscle cells on aligned nanofibrous polystyrene scaffolds and observed aligned actin filaments.¹²⁰ However, the authors point out that despite similar alignment as in porcine bladder tissue, the mechanical properties of aligned nanofibrous scaffold are still far different from actual bladder tissue, which exhibits two orders of magnitude greater ultimate strain (~ 500 vs. 2%) and a much lower elastic modulus (<0.1 vs. 16 MPa).¹²⁰ Stankus found that smooth muscle cells aligned with fiber direction for aligned scaffolds even in the presence of perpendicular moderate fluid flow.⁹⁷ Zong et al. aligned fibers by stretching random electrospun mats and found primary cardiomyocytes aligned with the scaffold.⁹³ Xu et al. observed the morphology of smooth muscle cells on aligned scaffolds and found a bipolar spindle shape indicating an appropriate phenotype capable of contraction. Actin and myosin filaments were observed for the contractile phenotype, which were absent for cells cultivated on standard TCPS.¹²⁶ An alternative approach to scaffold alignment is flow alignment.¹²⁷ Huang et al. has recently reviewed cardiovascular muscle tissue engineering including a discussion of mechanical and hemodynamic loading.¹²⁸

Electrical Properties

Leikes' group provides a rare example of incorporating electrical conductivity into nanofibrous composite scaffolds with work on electrospun polyaniline–gelatin nanofibers crosslinked after spinning.⁸⁴ Incorporation of polyaniline increased conductivity from 5×10^{-3} S/cm (gelatin) to 2×10^{-2} S/cm for 60:40 PANi:gelatin (volume). Cardiac myoblasts proliferated as well on composite scaffolds (of various ratios) as on pure gelatin fibers,⁸⁴ indicating that the addition of PANi for possible future electrical stimulation, which may be particularly important for cardiac tissue,^{98,129} did not affect biocompatibility.

Epithelial Tissue

Overview

Epithelial (or barrier) tissues include skin, tissues with basement membranes (i.e., bladders and blood vessels), and complex structures such as the esophagus. Generally they contain multiple cell types and have specific complex morphologies, for instance, attempts

to model blood vessels have utilized aligned, multi-layer scaffolds with differing fiber and pores sizes in each layer.¹³⁰ Endothelial cells are particularly important for temporary and permanent implants, such as bladder, vessel, or esophageal replacement where complete endothelialization prevents immune system response and blood clotting, which can significantly impede implant performance.^{83,131,132}

Surface Properties

For skin-like tissue engineering applications, modification of surface properties to enhance adhesion and proliferation of endothelial cells, skin fibroblasts, or keratinocytes has most commonly employed addition of natural biopolymers such as mixed fiber scaffolds with chitosan/PVA fibers,^{71,72} fibers from poly(ethylene terephthalate) (PET)/chitosan blends,¹³³ chitosan nanoparticle-coated PLGA fibers,⁷⁷ collagen-coated fibers,^{132,134,135} core-sheath fiber geometries with collagen sheath,¹³⁶ collagen blends,^{83,134,135,137,138} and grafting of fibronectin,^{85,139} or covalent bonding of gelatin to the fiber surface.^{140,141} Fibronectin-surface coating has also been employed for the hydrophilic polysaccharide, hyaluronic acid, blended with PEO.¹⁴² Effective coating by simple means, such as dipping, can be difficult. For collagen studies, core-sheath geometries allowed cell penetration into the scaffold, whereas collagen-dipped scaffolds seemed to retain collagen only on the exterior scaffold surface.¹³⁶ In subsequent papers, plasma treatment prior to dipping increased surface coverage allowing wetting of all fibers.^{132,143} In all cases (chitosan addition, collagen coating, grafting gelatin to the fiber surface etc.), enhanced adhesion, proliferation, and cell spreading were observed, compared with scaffolds containing only hydrophobic synthetic biodegradable polymers (such as PLA, PLGA, and PCL).

Materials such as collagen and chitosan are generally hydrophilic which may increase cell adhesion and proliferation; in addition, these natural materials may have specific surface characteristics that aid in cell growth. Kim et al. altered only hydrophilicity properties by mixing PCL and PVA fibers.¹⁴⁴ Attachment and proliferation of human prostate epithelial cells were also increased in this case (over PCL only), indicating that hydrophilicity alone can enhance cell growth. Park et al. modified the PLLA surface by grafting hydrophilic acrylic acid^{68,145} and found similar results. Spasova et al. tuned hydrophilicity with a PLLA/ poly (ethylene glycol) blend.¹⁴⁶ Although these modifications were only intended to alter surface hydrophobicity, Chua et al.¹⁴⁷ and other workers have hypothesized that modified surfaces might attract

(via electrostatic forces or specific chemical interactions) and immobilize biochemicals in solution, modifying the chemical environment at the surface and thus affecting cell attachment and proliferation.

Because of the different cell types and scaffolds involved, it is not possible to compare the relative importance of particular bioactive markers in collagen, gelatin, and chitosan over the effect of their water-retaining properties. As discussed in the general properties section, scaffolds formed entirely from these natural biopolymers exhibit dramatic swelling and poor mechanical properties even with crosslinking,⁸² motivating the need for a composite structure with both mechanical stability and a cell-appropriate surface. In fact, cell proliferation and morphology suffered in Kwon's study of PLLC–collagen blends for high collagen fractions (30 and 50%) because of swelling and shrinking in biological solution, whereas lower loading levels (5–10%) showed good results.⁸³ Venugopal et al. found collagen blend fibers (with PCL, <30% collagen) and pure collagen fibers to have similar effects on cell adhesion, proliferation, and morphology,¹³⁸ indicating that a well-designed composite can provide both mechanical and surface functions.

Min et al. utilized a chitosan nanoparticle-coated PLGA matrix to study the effects of various proteins from the ECM (type 1 collagen, fibronectin, or laminin) on cell adhesion and proliferation of keratinocytes (oral or epidermal) or fibroblasts.⁷⁷ Scaffolds were coated by an overnight soak in one of the ECM proteins. Cell response varied depending on substrate (PLGA only or chitosan/PLGA), cell type, and ECM protein. For instance, fibroblast cell adhesion could be enhanced by adding collagen or laminin coating regardless of substrate, but keratinocytes only experienced enhanced adhesion with the addition of collagen on the PLGA only substrate (laminin had no effect). Adhesion rates were similar for the chitosan/PLGA and collagen/chitosan/PLGA for keratinocytes. Laminin showed similar effects to collagen for fibroblasts; however, fibronectin showed no increase in cell adhesion for any cell type.⁷⁷ Zhu et al. studied fibroblasts and epithelial cells on PLLC with fibronectin or collagen surface modification, and found no changes in fibroblast attachment and proliferation for unmodified and modified scaffolds.¹³⁹ Zhu et al. did not characterize wetting properties of the PLGA scaffolds, but differences in PLGA and PLLA hydrophilicity for the particular co-polymer compositions used in these works, might explain the difference in results. Epithelial cells showed an increase in adhesion and the highest mitochondrial activity on fibronectin-coated substrates, whereas cell

density was lower with collagen coating and cells were 'clustered' rather than spread uniformly on the PLLA.¹³⁹ The authors hypothesize that because epithelial cells grow generally close to the basement membrane, which is fibronectin-rich, rather than near the collagen-rich ECM, the fibronectin-coated scaffolds may be more effective.¹³⁹ These results highlight the complex relationships between cell type, scaffold hydrophobicity, and specific cell–ECM protein interactions. For composite tissues such as skin, mucus membranes, and esophageal tissue, several cell types must organize and thrive on the scaffold surface, thus understanding both general conditions (such as hydrophilicity) and specific interactions (such as those with compounds found in the ECM) is important to continuing progress in tissue engineering.

One effect of a truly cell-compatible environment will be normal cell function, which can be determined in part by testing for natural by-products of the cell. Of particular interest may be the formation of a natural ECM by cells placed on an artificial scaffold. For instance, Zhu et al. tested collagen IV synthesis of endothelial cells on PLLC with or without a surface coating, finding that cells on a fibronectin-coated scaffold had enhanced collagen production (per cell) over those on non-coated scaffolds.⁸⁵ In micrographs, Mo et al. observed possible collagen fibrils between endothelial cells.¹²⁴

Patel et al. investigated *in vitro* wound healing on poly(L-lactide) nanofibers coated with heparin, which was then used to bind laminin (an ECM protein) and bFGF, both of which encourage growth and migration of dermal fibroblasts.¹¹³ An artificial wound was created in a monolayer of human dermal fibroblasts, with the underlying scaffold either random or oriented parallel or perpendicular to the wound. Random scaffolds provided adequate wound coverage after 48 h. However, wound filling was enhanced by perpendicular fiber alignment. In particular, the number of cells near the center of wound was significantly greater. For parallel fibers, cells were hindered in entering the wound region. These alignment effects were enhanced by the surface factors of bFGF and laminin.¹¹³

In a final example of surface modification, Chua et al. studied primary rat hepatocytes (liver cells) on electrospun scaffolds of poly(ϵ -caprolactone-co-ethyl ethylene phosphate), where the co-polymer is intended to tune the biodegradation rate of the PCL.¹⁴⁸ Hepatocytes form spherical aggregates in an appropriate environment with correspondingly enhanced cell function, such as urea and albumin production.¹⁴⁹ The scaffold surface was terminated with galactose,¹⁴⁹

which binds hepatocytes and encourages proper function. Attachment was enhanced by galactose coating for both nanofibrous scaffolds and films. However, while hepatocyte spheroids were tethered to the film substrates by only a few cells, leading them to detach relatively easily, cells penetrated the nanofibrous scaffolds, forming somewhat flattened spheres which encompassed the scaffold. Fibers within the spheroids appeared to degrade or be absorbed by the hepatocytes. Despite this change in spherical morphology, cell function, as measured by urea synthesis and albumin production, was maintained. Such a mechanically resilient structure with good cell function would enable an artificial tissue (i.e., a bio-artificial liver) which would be robust to flow forces, in contrast to 2D scaffolds where the spherical hepatocytes can be easily detached.¹⁴⁸

Chemical Delivery and Scaffold Degradation

As discussed in detail in the Section on Chemical delivery and scaffold degradation, one advantage that composite materials can provide is the ability to continuously deliver biochemicals. In particular, growth factors lose bioactivity quickly in solution and thus sustained release from a scaffold volume or surface is a particularly intriguing approach to obtaining functional tissue growth. Wei et al. utilized poly(lactic-co-glycolic acid) spheres seeded onto PLLA scaffolds to release platelet-derived growth factor. Cells exposed to the supernatant solution in which the scaffolds were soaked showed that bioactivity was retained.¹⁵⁰ Although not yet attempted for <1 μm sized fibers, an intriguing chemical delivery approach is to utilize DNA-containing particles as a component in fibers, which then transfect cells, causing them to subsequently produce particular growth factors (see for instance, Ref¹⁵¹). As pointed out by Lim et al., this technique overcomes limited bioactivity of, for instance, growth factors, in fibers or solution, and by altering the DNA type seeded throughout scaffold, might enable delivery of differing biochemicals to particular cell types.¹⁵¹

In addition to providing sustained delivery of quickly degrading biochemicals, chemical delivery via diffusion from encapsulated fibers can also deliver compounds that have poor water solubility. Chua et al. discussed chemical delivery of a lipophilic molecule, 3-methylcholanthrene (3-Mc), which stimulates cytochrome enzymatic activity in hepatocytes.¹⁵² Lipophilic biochemicals are particularly difficult to incorporate into aqueous environments and require emulsions where droplets of organic solvent are suspended in the aqueous solution. Chua et al. utilized a two-layer system where hepatocytes migrated through

a few fiber thick mat of 3-Mc doped fibers to attach to fibers surface-modified with galactose to ensure adhesion (see surface modification section above). Surface functionalization required exposure to ultraviolet light, which degrades poly-cyclic aromatic hydrocarbons such as 3-Mc, preventing incorporation of both functionalities into the same fibers. Cells grown on the bilayer fibers showed enhanced cytochrome activity, even in comparison with cells exposed to 3-Mc via solution (in emulsion). The authors hypothesized that direct contact between the cells and 3-Mc-containing fibers enabled a non-aqueous transfer path from the fiber through the cell membrane. This conjecture was supported when cytochrome function decreased significantly after a porous membrane preventing cell contact with the 3-Mc fibers was placed between the two fibrous layers.¹⁵²

Mechanical Properties

Several authors have discussed ideal mechanical properties for barrier-type tissue reconstruction. Zhang et al. points out that the mechanical properties of the scaffold should be similar to that of the ECM, so cells can penetrate into the interior of the scaffold through openings similar to or smaller than cell diameters, by pushing into the pores.¹³⁶ Similarly, Zhu et al. selected PLLC co-polymer for work toward artificial esophageal material, with a glass transition temperature of 9°C so that the scaffold would be elastomeric at biological temperatures.⁸⁵ They compared the mechanical properties of collagen-coated PLLA co-poly(ε-caprolactone) with Dacron (commonly used for vascular grafts) and actual coronary artery, finding that the tensile strain of the nanofibrous matrix was closer to that of the coronary artery than to the Dacron (which has a much higher tensile strain) with a comparable ultimate strain.¹³² The authors argued that such a nanofibrous material might be more suitable for artery patching and replacement. They attribute the ultimate strain (175%) to alignment of the random fibers under stress, with a simultaneous breaking of fibers.¹³²

Because barrier tissues contain and support multiple cell types and serve complex roles, high levels of multifunctionality are needed. For instance, Kwon and Matsuda used a collector that periodically moved between two electrospinning sources to mix fibers with improved surface properties (via collagen blending) and drug delivery capability (doped with heparin) to encourage endothelialization and discourage coagulation for vascular grafts. The base polymer (PLLC) was chosen for its elastomeric mechanical properties.⁸³

Tuning electrical properties with composites to better facilitate the growth of functional epithelial tissue (within the constraints of a nanofibrous scaffold) has not been reported.

Connective Tissue

Overview

On the basis of the function of providing rigidity and support in the skeletal system, bone tissue engineering has seen the most progress in regenerative medicine, with the implantation of fibrous nanocomposites seeded with progenitor- and lineage-specific bone cells (osteoblasts) to form a functional tissue.¹⁵³ Connective tissues typically display a very large amount of ECM materials.

Surface Properties

In a composite reported by Venugopal et al., PCL provided mechanical stability, collagen supported cell adhesion and proliferation, and nanoHA (nHA) provided the mineralization of osteoblasts for bone regeneration (chemical modification in our scheme). The addition of nHA and collagen increased osteoblast proliferation rate by 35% and mineralization by 55%.⁷⁴ In another example, Type I collagen and poly(3-hydroxybutyrate-cohydroxyvalerate) (PHBV) were electrospun by Meng et al. PHBV is a well-known biodegradable, biocompatible, nontoxic, thermoplastic polyester produced by bacteria. The study showed that the PHBV-collagen nanofibrous scaffold accelerated the adhesion and growth of NIH3T3 cells more effectively than PHBV nanofibrous scaffold.⁴⁹ By incorporating gelatin with PCL, bone marrow stromal cells spread better and migrated deeper inside the gelatin/PCL scaffold. The cells spread and stretched themselves on the composite in contrast to a round shape exhibited in pure PCL scaffold. The cells infiltrated up to 114 μm in gelatin/PCL scaffold compared to 48 μm in only PCL scaffold.²³

In an example of tuning surface area, research performed by Sahoo et al. investigated the supplementation of PLGA nanofibers on knitted PLGA scaffold for tendon/ligament tissue engineering.¹⁵⁴ Knitted PLGA displayed the appropriate mechanical properties and integrity for replacing a tendon/ligament while PLGA nanofibers possess a larger surface area and better hydrophilicity. Thus, depositing nanofibers on a knitted substrate would facilitate cell attachment, new ECM deposition, and tissue formation without compromising mechanical integrity. Porcine bone marrow stromal cells exhibited a higher expression of collagen

I, decorin, and biglycan genes as well as an abundant production of ECM on the nanofiber-knitted scaffold.¹⁵⁴

Chemical Delivery and Scaffold Degradation

The ability to incorporate sensitive growth factors and particles into biomaterials during the fiber formation offers important benefits for chemical delivery of these factors during tissue-specific formation. For bone formation, bone morphogenetic proteins (BMPs) play an important role in osteogenesis and bone metabolism.⁴⁶ However, exogenous administration of BMPs in buffer solution gives problems such as rapid diffusion from the initiation site and the loss of bioactivity, which leads to insufficient local induction and hence failure of bone regeneration. A tissue engineered scaffold that controls the release of BMPs by adequately immobilizing them is required. However, the capability to control the release kinetics of BMPs is difficult when they are physically adsorbed into the fiber matrix.⁵¹ rhBMP-7 was encapsulated into PLGA nanospheres immobilized on a nanofibrous scaffold for controlled delivery and examination of ectopic bone formation via subcutaneous implantation in rats.⁸⁷ Bone formation occurred consistently on the surface of the BMP-encapsulated scaffolds while a scaffold coated with adsorbed BMP had poorer results. The incorporation of BMP into the fibers protected biological activity and provided local delivery with prolonged duration to induce ectopic bone formation throughout the scaffold. Both initial BMP levels and sustained local release are important for achieving adequate bone induction and mineralization.⁸⁷ The BMP was released into the body as a result of combination of diffusion and degradation mechanisms with the BMP-encapsulated scaffolds. The release times could be varied from weeks to months by varying the molecular weight of PLGA.

To address the biphasic nature of mineralized connective tissues, scaffold design has focused on porous polymer/inorganic materials,⁷⁶ including incorporation of chemical signaling agents. Inorganic materials include the encapsulation or surface localization of HA, calcium phosphate, bioglasses, and phosphate glasses. These materials are selected to match the chemical composition, cell-bonding ability, and bioactivity generated upon degradation of mineralized tissue and can be incorporated into the polymer solution prior to electrospinning. HA, a bio-ceramic component, possesses the effective affinity for regulating cell function and promoting osteogenesis and mineralization.⁷⁴ However, HA can only be used in its single-component form in the form

of powders, granules, or coatings as it is extremely brittle. Introducing the polymeric component overcomes the brittleness.^{64,74,155} When osteoblasts and fibroblasts were cultured on PCL–HA fibrous scaffolds, they appeared well expanded and attached on the fiber surface after about 1 h.^{64,74} Alkaline phosphatase activity increased with increase in HA wt%, as did fiber diameter.⁶⁴

Mechanical Properties

The challenge for connective tissue (particularly bone) is to provide adequate mechanical strength prior to cellular deposition of a natural ECM. Kelly et al. have predicted the optimal mechanical properties for osteochondral repair with values for immature bone to cortical bone from 1000 to 17,000 MPa, respectively.⁵⁹ Silks are attractive biomaterials for bone tissue engineering because of their biocompatibility, slow degradability, and excellent mechanical properties. Nanofibrous scaffolds have been made from silk fibroin by combining the unique structural features generated by electrospinning (possible by blending with PEO) with functional factors, such as BMP-2 (BMP-2) and nHA particles.⁵¹ Bone formation was assessed by growing human bone marrow-derived MSCs *in vitro*. The incorporation of BMP-2 and nHA in the scaffolds resulted in a four- and sixfold increase in calcium depositions (chemical modifications).⁵¹

Optimization of electrical properties for tissue scaffolds designed to support connective tissue cells in nanofibrous geometries has not been reported.

Connective Tissue Conclusions

The bone matrix is intimately associated with different types of soft connective tissues, muscles and nerves, and thus must effectively interface with them. Kim et al. have suggested that a membrane structure with a composition gradient from one side to the other can guide tissue regeneration so that it proceeds more effectively and specifically such that interfaces between tissue can be tuned and perhaps, different tissues grown on the same scaffold.⁸⁹ Regenerative potential for both soft and bony tissues can be optimized with such a membrane by stimulating progenitor cells to be differentiated into specific types of cells and thereby inducing further matrix production and wound healing. Electrospinning would have the advantage of allowing the layers to be realized so that a composition gradient is produced in the membrane structure by simply changing the composition of the solution. However, the designing of the layered structure appropriate to specific applications remains a future area of research.⁸⁹

Stem Cells

Overview

Although most of the work reviewed here has focused on establishing multifunctional composite structures to result in functional tissue from particular differentiated cell lines (e.g., endothelial cells, smooth muscle cells, or osteoblasts), an alternative approach is to seed pluripotent cells, such as adult MSCs or other progenitor cells, and force differentiation down a particular cell path by directing the lineage of the cell via specific stimuli from the scaffold and/or the chemical and mechanical environment. MSCs are used particularly for tissue engineering applications because of their multilineage potential, source tissue availability, and ease of retrieval. Many groups have focused on determining these chemical and physical triggers that determine differentiation. These stimuli can be categorized into either inductive soluble factors, the scaffold's physical properties or chemical functionality, and the application of physical stimuli such as fluid shear stresses or electrical fields.

However, in order to maximize the efficiency of a stem cell approach, cell culture environments that preserve cell pluripotency, to increase the number of progenitor cells without differentiation, must also be developed. For instance, Xin et al. showed that PLGA nanofibrous scaffolds maintained hMSC phenotype in the absence of chemical signals to differentiate into osteogenic or chondrogenic lines.⁶⁶ Furthermore, Li et al. observed human embryonic palatal mesenchymal cell proliferation on crosslinked natural biopolymer nanofibers of gelatin, elastin, collagen, and tropoelastin,⁸² and Moon et al. tested MSC on gelatin/PLA nanofibers,¹⁵⁶ although no test for retention of pluripotency was noted in either case. Research on developing such plasticity-preserving protocols is the subject of this section. This work has focused primarily on surface modification with no current reports of mechanical, chemical, or electrical stimulation (within a nanofibrous composite).

Surface Properties

Chua et al. studied scaffolds of electrospun poly(ethersulfone) surface modified with amine, hydroxyl, or carboxylic groups and their effect on hematopoietic progenitor cells from cord blood.¹⁴⁷ Amine fibers were found to maintain pluripotent cells (allow cell reproduction without differentiation) more effectively than unmodified, hydroxyl- or carboxylic-terminated scaffolds. In addition, amine-terminated nanofibrous scaffolds outperformed films with the same amine surface functionalization, indicating that both surface modifications and morphology were

important. Comparing fibrous scaffolds, cells on the aminated scaffolds were well adhered, whereas cells were easily washed from unmodified fibers and those with other surface treatments. Circular cell colonies (100 μm to 1 mm in diameter) were observed on the aminated scaffolds with little cell–cell contact, which may indicate a radial growth pattern with good cell adhesion but also mobility. Multiple attachment points from cells to fibers were observed. Such colonies were not observed on amine-treated films and cells were dense primarily near film folds or crevasses. The authors hypothesized that the positive charge on the amine might interact with the negatively charged CD34 receptor on the progenitor cells and such binding trigger particular cell types (pluripotent or differentiated into particular line) upon proliferation. An alternative possibility is that the positively charged amine surface immobilized biochemical, in particular, cytokine, from the solution phase, modifying the chemical environment near the scaffold and thus influencing cell differentiation.¹⁴⁷ In a follow-up study, amine-terminated nanofibrous scaffolds with differing hydrocarbon chains linking the amine and surface (i.e., ethylene, butylene, and hexylene) were tested, again with cord blood progenitor cells.¹⁵⁷ Similar results were obtained with increased adhesion, decreased differentiation upon proliferation, and cell colonies observed for all three amine surface treatments.¹⁵⁷

Beyond the scope of the review, Cetinkaya surface-modified polyester non-woven fabric with leukemia inhibitory factor, which is used in solution to prevent differentiation, and found it effective in allowing pluripotent propagation for embryonic stem cells when surface-immobilized.¹⁵⁸

CONCLUSION

The use of fibrous nanocomposite scaffolds for tissue engineering is rapidly expanding. In fact, no doubt many reports will have appeared before this review is published. However, in reviewing the current literature, the authors identify the following areas (certainly not an inclusive list) that seem to require particular attention by the material scientists and biomedical engineers working in this field and are topics of growth for the future.

More Effective Chemical Delivery, Particularly of Growth Factor

In order to further functionalize artificial tissue scaffolds, research will need to be performed that characterizes the presence, effect, and spatial

expression of growth factors in tissue defects. Future scaffolds should possess the ability for controlled release of soluble factors by cell interaction alone, and not merely by degradation or diffusion mechanisms. Temporal and spatial distribution of the rate of controlled release is paramount as this occurs in natural defect repair. Also, the ability of the seeded cell population to remodel the artificial scaffold and synthesize their own ECM will continue to be a challenge.

Understanding of the Importance of Electrical Properties

As is clear throughout the review, this is an under-explored area, despite the fact that natural biopolymers, such as collagen, have both piezoelectric (a correlation between voltage and physical dimension) properties (when dry) and non-zero ionic conductivity ($\sim 10^{-3}$ S/cm in aqueous solution). Piezoelectric effects are also seen in bone, and such phenomena as streaming potentials and opening and closing of ion channels are known to be important in cell signaling, not to mention the known electrical signals in cardiac and neuron tissues. Future studies should attempt understanding the role of endogenous ionic currents and how small fluctuations in this current can influence cell membrane potential, DNA synthesis, and ultimately gene expression.

Increased Specificity and Reactivity

The ECM is complex and requires biomaterials that are more interactive and tissue specific. Furthermore, natural and synthetic polymers alone will not be able to recapitulate the developmental biological side of the tissue; composite systems offer a method to incorporate multiple functionalities and impart desired properties for a material. Some researchers have taken this initiative by quantitatively measuring how the mechanical properties of bone change at a fracture site during healing. These changes and the reorganization of the ECM demonstrate how responsive and dynamic the ECM must be for healing and regeneration to occur.

Influence of Scaffold Properties: Surface Chemistry and Substratum Elasticity

Precise control of the properties of artificial tissue scaffolds can dominate the results of stem cell differentiation and the gene expressional profiles of their progeny.^{31,92} Works by Engler et al. and Curran et al. were able to distinguish large morphological and gene expression differences in regard to the properties of the substratum that MSCs were cultured

on; yet these works are merely an introduction of what can be achieved through the tailoring of scaffold properties. As tissue engineering scaffolds continue to become more tissue specific, multifunctional scaffolds with varying chemistries and physical properties will

perhaps be able to control the persisting issue of interfacing two different tissues together such as muscle to bone, or bone to cartilage. The control of these interfaces will likely result in the true success of an engineered tissue.

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