Chapter 7
Polymers in Tissue Engineering

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Abstract The landscape of polymer selection and processing techniques is constantly evolving in the field of tissue engineering and regenerative medicine. This chapter will cover new advances in polymers that are used to regenerate functional tissues used to repair or replace tissues lost to age, disease, injury, or congenital defect. The focus will be on new processing techniques and the incorporation of biologics or drug delivery to enhance cellular response and ingrowth into the polymers that will create a more functional tissue replacement by engineering the polymer tissue interface. Special emphasis is placed on new frontiers in tissue engineering the lung and liver.

Abbreviations

CT	Computer aided X-ray tomography
E′	Storage modulus
E″	Loss modulus
ECM	Extracellular matrix
ELISA	Enzyme linked immunoabsorbant assay
HAG	Hydroxyethyl methacrylate-alginate-gelatin
MGLA	Modified gelatin sponges with lactobionic acid
PCL	Poly(carprolactone)
PCL-U4U	Polycaprolactone bisurea

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PDMS	Polydimethysiloxane
PEG	Poly ethylene glycol
PLA	Poly lactic acid
PLGA	Poly lactic co-glycolic acid
PLLA	Poly-l lactic acid
TLR4	Toll-like receptor 4

Introduction

While there are many polymeric biomaterials used in medical applications, many of which are covered in other chapters of this textbook, we will limit our focus to tissue engineered polymer containing constructs that either 1. Contain a polymeric scaffold + cells prior to implantation or 2. Contain a polymeric scaffold with a bioactive factor to elicit native cellular recruitment into the scaffold or drug delivery to form a functional tissue replacement. This book chapter is not meant to be all encompassing. Rather, it is to provide an up to date view of the status of new breakthroughs and in the field of polymers in tissue engineering. This chapter focuses on aspects important to the engineering and design of polymeric biomaterials for use in functional tissue regeneration.

Market reports estimate a steadily increasing market for tissue engineered products through 2016 at an $85 billion dollar market worldwide. While some polymers + cell products have been on the market for over a decade (mostly in the wound healing arena), there are emerging areas wherein more polymeric tissue engineering products will likely move to the marketplace. These emerging areas are likely in tissue engineered blood vessels, neurological regeneration, and hydbrid assist devices for the lung or liver. The general concepts of tissue engineering with polymers are shifting focus from the polymeric scaffolds to not merely be a structure, but to become an active template to guide cellular growth and differentiation. This emerging understanding of cellular signaling in response to polymeric matrices is shaping the field of tissue engineering. Material science technology advances in arenas of three dimensional bioprinting, new hybrid biopolymers, and complex microfluidics will also continue to shape the field.

Polymer Processing Techniques to Mimic Tissue Architecture and Strength

Proper tissue growth, function, and adaptation have as much to do with the architecture of the extracellular matrix as it does with the cells that populate the tissue. Skin without elasticity is a strait-jacket. A lung without branching bronchi is a wet paper bag. Bone without intricately-connected canals through concentric layers of hydroxyapatite and buttressed by trabeculae is a ceramic coffee cup waiting to be smashed. The biological polymers which form the shape and scaffold of the organ

cells are not merely blank shelves; the very architecture and mechanical properties of the polymer scaffold signal the cells on them to perform specific tasks and grow to a specific form within the confines of a specific scaffold. When the scaffold architecture fails to contain the cells on them, free-floating blobs of bio-matter can form thrombi, cysts, or tumors. When the scaffold mechanical properties fail to fit the requirements of function, the cells react by reacting with inflammation or scarring that destroy the function. And when mal-processed scaffolds fail to provide the proper signals to the attaching integrins of the cell, the health and shape of the cells deteriorate in like-step with the engineered organ as a whole.

This section addresses the current polymer processing techniques used to create engineered extracellular matrix (ECM) for cells that provide the appropriate architecture and mechanical properties for cells, while maintaining the appropriate embedded cell-signaling molecules. Each material and processing method (gelation, heat or high voltage extruding, printing, or biological formation) each have their own strengths and weaknesses. We will also briefly cover current strategies in accounting for material processing weaknesses by combining techniques.

Hydrogel Polymers with Self-Assembled Nanostructure

The versatility of hydrogels makes them one of the most popularly studied and medically applied polymers in the tissue engineering field. Hydrogels, which are polymers suspended in water, are apt for perfusing with hydrophilic nutrients and growth factors that will diffuse to the residing cells. Because the material is often processed from an aqueous state, they can be physiologically safe for suspended cells during and after the processing of the material which takes it from viscous fluid to gel. The hydrophilic diffusion and aqueous beginnings of this polymer make it uniquely suited for encapsulating cells deep within the scaffold from the initial constitution of the polymer. The ability to be injected also give hydrogels a unique advantage over numerous polymers in clinical applications where surgery would like to be avoided.

However, it is worth noting that although numerous organs have a soft and flexible consistency, only a few tissues (such as bone marrow or the nucleus pulposus of the intervertebral disc) have mechanical properties comparable to hydrogels. When compared to most tissues, hydrogels have numerous mechanical compatibility issues which are related the material's fabrication and processing. Hydrogels can break apart due to stresses such as compression or sheering, their ability to degrade has to be balanced to their physiological function, their ability to diffuse nutrients and factors is difficult to regulate, and controlling those factors often sacrifice biological functions. The mechanical properties of hydrogels are measured with storage modulus $\mathbf{E}'$, the amount of energy a gel can store as elasticity without permanent deformation, and the loss modulus $\mathbf{E}''$, the energy dissipated as heat. For example, a sheep's nucleus pulposus has a mean storage modulus of $E' = 64{,}000$ Pa, and a loss modulus of $E'' = 23{,}000$ Pa [1]. A gel with the bare minimum of practical use has a

storage modulus of at least $E' > 1$ Pa [2]. To address those issues, there are various molecular designs and hydrogel processing techniques.

Picking-up on one of nature's own methods of assembly, self-assembling peptides are amino acid sequences which use the charges of the side groups to attract another strand and form strong van der Waal interactions. One of the simplest strategies is to use strands of alternating sequences of hydrophobic and hydrophilic amino acids which form β-sheets [3, 4], one of the major secondary structures in a protein. Repeating sequences of peptides can also form bonds with itself to form α-helices hydrogels [5]. Peptides can also be tethered with other non-amino segments, such as hydrophobic carbon chains, to form polar molecules with a hydrophobic and hydrophilic side. These amphiphilic molecules assemble in a similar fashion to a micelle or liposome and form hydrophilic fibers with hydrophobic cores [6]. With peptide-based gels, modifying the amino acid sequence affects the inter-molecular bonds. For example, basic physics tells us that positive charges are attracted to negative charges, and vice versa. Positively charged amino acids such as Arginine (R) and Lysine (K) are patterned with negatively charged residues such as Aspartic Acid (D) and Glutamic Acid (E) on the same or differing peptides. Varying the quantity and pattern of repeating ionic amino acid residues will change the mechanical properties [7]; however, longer peptides does not necessarily mean stronger peptides [8] so a proper balance between interacting molecules and unwieldy size must be struck. Much like varying the quantity of charged amino acid residues, varying the length of the hydrophobic and hydrophilic components of an amphiphilic molecule will change the degree of interaction between molecules, and vary the mechanical properties and structure of the hydrogel [9, 10]. The concentration of the peptide in solution will also affect the amount of molecular interaction, and thus an increase in peptide concentration will usually increase the storage modulus of a hydrogel [4, 11]. Because hydrogels initially start as a suspension of molecules in a viscous solution, the final result is usually a haphazard arrangement of fibers and molecules distributed by Brownian motion. One molecular level is to use electromagnetic fields to process the material. Peptide hydrogel fibers have been shown to align along a magnetic field [12]. This provides physical cues at the molecular level to proliferating cells.

The degree of interaction between peptides is also affected by the ionic environment. Changing the pH [4] or concentration of ionic salts [13] in solution will also affect the inter-molecular bonds between the peptides, modifying the mechanical properties of the hydrogel. A simple demonstration of this principle is the isoelectric point of a protein, where at a particular pH a peptide will lose charge (like and repulsive charges) and aggregate together. Temperature also affects the mechanical properties of gels. Not only can gelation be induced by change in temperature, the temperature at which gelation is induced (by alternative means such as pH or concentration) affects that mechanical properties of the material [14].

In most biological circumstances, a physiological pH, ionic balance, and temperature is needed for cell culture in a hydrogel. So while this usually doesn't affect tissue culture, which is usually at physiologic conditions, this is important to remember when you are testing mechanical properties outside the culture environment.

Chemical cross-linkers and photo-sensitive chemical processes can also cross-link peptide hydrogels to initiate gelation and strengthen bonds. Ultraviolet light breaks peptide and other bonds which then re-form, but the biological function of the modified material must be tested due to the random nature with which the bonds are broken. Chemicals such as gluteraldehyde are strong cross-linking agents with a known toxicity, and thus alternative "natural" cross-linkers such as transglutaminase and genipin are being investigated [15].

By modulating properties such as temperature and pH to maintain the solution-state, the concentration of the hydrogel's solutes can be increased without inducing gelation. The temperature or pH can then be modified back to the desired state, and the hydrogel formation will have a concentration or property that may not have otherwise been attainable. However, the process should be careful not to permanently denature the peptide during heating or changing pH, as the molecular or biomechanical properties may not be what were expected if the peptides are damaged by high temperature or variation from neutral pH. Another strategy for inducing gelation is mixing different combinations of similar peptides. A peptide sequence with a biological function may not be conducive to forming a gel, but when combined with a similar peptide with stronger mechanical properties the combination can have intermediate qualities in mechanics [2] and biological activity.

Spun, Extruded, and Crosslinked Polymers with Arranged Nanostructure

Even though most natural biological polymers are microscopic, with dimensions on the order of nanometers, they often assemble into a macroscopic structure that can be seen with the naked eye. The connective tissue of muscle, tendons, cartilage, and skin can appear stringy when stretched and teased apart. Even bone, lung, nerve, and other tissue have apparent millimeter-scale structures which belie the fact that their true functional units are really much smaller.

Extrusion and spinning are material processing methods that long-predates micron-level materials engineering. The simplest example of extrusion is pushing dough through a press to create strings of noodle-like polymers. Spinning is best exemplified by exerting centripetal force on melted sugar to form cotton candy. In both examples, there is a force pushing the material in one direction (either a press, a spinner, or even an electromagnetic force), and another force pulling the opposite direction (the mold or molecular forces). When a balance is achieved between those pushing and pulling forces, the material forms a fiber which can resemble the stringy material found in tissue. These macro-scale materials can very closely match the topography and mechanical properties of biological tissue, including porosity for the diffusing nutrients and isotropic or anisotropic strength (force-bearing in single or multiple directions). The modern challenge of tissue-targeted polymer engineering is incorporating the unseen physical cues of nano-scale components (such as collagen fibers) of the milli-scale materials (a collagen fibril). Beyond the mechanical properties,

incorporating biological components are also challenging; the solvents and environments used to process the polymers for extrusion and spinning can be corrosive to the organic material. Synthetic polymers are also fundamentally problematic because the most common and versatile polymers are almost exclusively hydrophobic, a property which cells and other biological components respond to poorly. Fortunately, there are strategies to overcome those issues and more-closely mimic natural tissue.

Electrospinning

Electrospinning as a material fabrication process has existed for decades to create a densely-woven mesh of fibers. What makes this process unique is that the fiber diameters achieved by electrospinning are much smaller, down to several nanometers in diameter, compared to other synthetic polymer synthesis methods. New and creative means to applying the electrospinning process has led to numerous potential applications for tissue engineering. The process resembles extrusion in that the material in solution is usually pressed out of a syringe before forming a fiber. However, the process of using a syringe is optional (for example, rolling drums of polymer can be used for mass-production methods [16]), because the true material "extrusion" comes from the electromagnetic force on the solution which pulls molecules out of solution and towards a collection site. As the solvent evaporates around the solute, the molecules of the polymer pull with it a chain of polymer which forms the deposited fibers at the collection site. If there is too much electric force, too little molecular attraction between the solute molecules, too much distance for the polymer to travel, or not enough polymer at the site where the solution is being charged, the fiber becomes discontinuous or just deposits as droplets at the collection site. The rate at which polymer is placed into the field of electricity (often controlled by a syringe pump), the distance between polymer source and collection, and the environmental conditions such as temperature and humidity all affect the mechanical properties of the electrospun fibers [17]. The type of polymer, the solvent, and the concentration vary greatly. The method of collecting the fibers serve as one of the final processing steps for electrospinning which determines mechanical properties, and these methods vary greatly.

The classical method of collecting electrospun fibers is to place a grounded sheet of metal opposite the charged source with the polymer. This creates a dense, isotropic (all-directional), mat of fibers. This structure tends to lack space between the fibers, and important feature if cell migration into the material is desired, but strategies such as adding dissolvable fibers [18, 19], leaching inorganic salts [20, 21] or other particulates [22], or even jets of air [23] at the site of collection mechanically create some space for residents cells on the material to grow into. However, while many human tissues have significant thickness and require a length and depth of cells on an extracellular matrix, it is important to consider that many tissues (such as epithelial cell-based tissue) are single-layered, and a basement membrane which isn't penetrable by cells is important for proper morphology of attached cells [24].

Collection doesn't have to be a static process. By rotating the collector in the axis perpendicular to the incoming fiber at increasing velocities, the direction of the fibers collected increase in their alignment perpendicular to the axis of rotation [25]. Put simply: the faster the collector rotates towards the polymer source, the more aligned the collected fibers are in the direction of rotation. This is key for a number of tissues that have anisotropic (single-directed) properties such as connective tissue and nerves, as the direction the fibers are arranged serve as the guide for the proliferating and extending cells [26, 27].

When processing the electrospun fibers into suitable scaffolding for cell culture, the forces don't have to be applied as the fibers come to rest. Strategies for manipulating the fibers mid-air have been devised to create more complex structures. Manipulation of the electric field by using curved collection plates and point-probes can create turbulence as the fibers approaches the collection site, causing the electrospun fibers to tangle before coming to rest [28]. The result is a material which is not packed as tightly as sheets of fibers and resembles a cotton ball. Air-collecting electrospun fibers can also produce aligned scaffold between conductive points [29] or directed by a combination of blower and electric fields to a non-conductive target [30].

The fibers themselves can be modified significantly in the way they are processed. The emulsion of immiscible liquids which are then electrospun creates fibers with a core of one type and a surface of another type. If the internal material is dissolvable, it creates a hollow core [31]. And additives to the polymer do not have to be removed. Blends of inorganic and organic material, which can be purified [32] or heterogenous extracellular matrix components [33], can add bioactive components for interacting cells or change the material's mechanical and chemical properties.

The choice of material for any materials processing method can lead to a dilemma with the choice of solvent. In electrospinning, the polarity (polar or non-polar), relative permittivity (or dielectric permittivity, the force between two points in a material), conductivity, and viscosity affect the fiber diameter of the final product [34, 35]. Materials such as polycaprolactone, poly-lactic acid, and polyethylene oxide are generally very soluble in any organic solvent, but more complex or organic materials such as collagen may have a smaller range of acceptable solvents [36], and the types of solvents used may be harsher on the material's final biological composition. While there are some examples of non-toxic solvents used for biological materials [37], any solvent should be fully dried via desiccation or other means to ensure the removal of solvent. Sterilization procedures, whether it is ethanol washing or other biochemical processes, should also consider the effects on the final material's mechanical and biological properties [38, 39].

Extruding and Printing

Melt extrusion is a cost-effective means of producing a number of structures, and for applications in science and medicine melt extrusion is useful in pharmacology with drug delivery [40, 41]. However, achieving the nano-scale detail in

architecture is difficult with this method compared to other processes, but there are other advantages. With the explosion of affordable home desktop 3D printing options (either home-made or commercial), it was inevitable that tissue engineering applications would be found. But even before the recent advances, the paper-thin process of ink printing was just as applicable when printing sheets of cells. Synthetic materials can be printed as melted extrusions or powders with binder. Printing biomaterials with or without cells involves computer-controlled positioning of a nozzle connected to a material source composed of liquid or hydrogel and guided to a target either by gravity and charge (such as in Fluorescent-Assisted Cell Sorting) or by laser (remember that photons can impart a force). The end result is controlled positioning of biomaterial and/or cells in a pattern that can mimic the tissue of choice. Two challenges facing this method is what would be expected from any type of prototypical printer: accuracy and resolution. Also, because natural tissue is interconnected, connecting the individual depositions of biomaterial to accurately impart mechanical properties is another challenge to be tackled.

Creating an anatomically-shaped scaffold is relatively simple for a 3D printer. A computer-aided X-ray tomography (CT) of a patient's bone can be converted to a 3D model and replicated as porous melt-extruded polycaprolactone [42] in a day. Fortunately, materials such as porous PCL has similar mechanical properties (compressive modulus and yield strength) to trabecular bone [43]. Because bone tissue grows heals itself when set properly, bone tissue engineering appears relatively forgiving of shortcomings in nano-structural material characteristics. Powders deposited by a 3D printer can also create shapes that are bioactive and have a range of mechanical properties dependent on the pore sizes and structures created; however, the ceramic-like materials powders such as calcium phosphate often produced tend to be very brittle and not suited for load-bearing [44]. Improvements to the flexural strength and bioactive properties in printing of calcium phosphate powders has been made by adding collagen and an emulsifier to the binder [45].

Biologically Generated Polymers

Synthetic materials and the processing that goes with it give the tissue engineer a degree of control over the process; however, there are still many things that cells construct better than our best designed equipment. Decellularized tissues have been used as implants for bone, tendons, ligaments, and heart valves for many years now. Some grafts are wholly-accepted by the body and incorporate the patient's cells. Other grafts, such as heart valves, have to be processed in a way that destroys the biological signaling in order to serve their mechanical function and never integrate into the recipient. Using decellularized organs as the biopolymer for tissue engineering come with a number of advantages: the remaining polymer is mechanically and morphologically similar to the original organ, the polymer has bio-active molecules matching what the native tissue would expect.

However, the availability of human-sourced tissue is limited and potentially controversial, and animal sources can have important differences compared to their human counter-parts.

Tissues are decellularized using a several different approaches, sometimes in combination with one another, which effectively remove the overwhelming majority of all cells and cellular material from the extracellular matrix architecture. These techniques include the use physical techniques such as snap freezing or mechanical agitation, and chemical techniques such as using ionic or non-ionic detergents, zwitterionic detergents, basic or acidic solutions, and hypotonic or hypertonic solutions [1]. Depending on the methods used the leftover scaffold will retain mechanical components (elastin, collagen), natural binding sites, and growth factors in different quantities; some decellularization methods are better at retaining certain components over others [1–3]. Equally important to the methods or solutions used for decellularization is the process used to expose the intact tissue. Tissues composed of complex architecture or expansive vasculature will most likely benefit from a perfusion decellularization system where the vasculature is perfused with detergent as well. This works especially well with lung decellularization where the pulmonary artery as well as the trachea can be cannulated [4, 5]. Thin tissues such as bladder or skin can be decellularized using an immersion process where the tissue is suspended in the decellularization solution [6]. Thick tissues or organs can be decellularized using immersion processes as well; however they must first be cut into thin strips. Both immersion and perfusion techniques can benefit from constant flow or agitation provided from a decellularization bioreactor [4, 7].

To exploit the biochemical benefit from naturally-derived polymers, it isn't always necessary to wholesale incorporate the entire molecule. Specific peptide sequences from laminin such as RGD [46–49] and YIGSR [46, 48, 50] or from elastin like VAPG [51] have been isolated as cell-interacting sequences. These molecules can be chemically tethered to synthetic polymers by means such as carbodiimide chemistry [46], click chemistry [50], or custom-designed by synthesizing peptides with Fmoc chemistry [2]. Biologically derived polymers don't have to be for cell attachment, but for other enzymatic processes such as degradation if cell-regulated metalloproteinase cleavage is desired [2, 8]. While this doesn't directly affect the mechanical properties or architecture of a polymer scaffold, it does allow for greater biological activity of polymers that may have desired mechanical and architectural features you can't achieve with biologically-derived polymers.

Combining Techniques

It is unlikely that a single solution can be found to solve the problems of tissue engineering. Our own organic tissue is not merely deposited by cells, but requires techniques to compartmentalize the material, mechanical and biological signals to direct the orientation, and particularly arranged charges within specific environments to induce assembly of the raw biological material. A truly biomimetic

approach to tissue engineering involves incorporating multiple polymer processing techniques to form the desired shape, micro-architecture, strength, and cell interaction needed to form tissue just as nature intended.

For example, while electrospun nanofiberous material of synthetic polymers can be mass produced and have a tune-able properties, they often lack biological motifs and hydrophilic chemistry that a cell is accustomed to. Hydrogels and biological polymers can be much more similar to the cell's native environment, but far more difficult to tune mechanically. However, by coating an electrospun scaffold with peptides in hydrogels new biological properties are given to the electrospun scaffold, and new mechanical properties are given to what was originally just a hydrogel material [48].

While decellularized organs to produce ECM provide ready-made scaffolding, the end result might not be the ideal material for the purposes. Instead of a porous organ, a hydrogel encapsulating the cells may be more feasible for culture. However, taking an example from hydrogel synthesis and processing, ECM after processing by proteases can reform as a hydrogel, providing the cell-interactive motifs and growth factors of normally-decellularized ECM [52].

The use of a method for fabricating a material doesn't have to be used exclusively for fabrication. While 3D printing is known for producing a material or object from the ground-up, the method can also be used to print on and over other materials. An example of this is using a 3D printer to deposit hydrogels onto a mold, and then use the printer to apply cells [53].

Fusing the spatial customization of 3D printing with the fiber diameters of electrospinning, short-range high-voltage polymer extruding through a computer-controlled nozzle across a high voltage electrical field can create customizable micro-fiber structures with controlled pore sizes [54, 55]. While not quite yet at a level where printed nano-resolution structures are a reality, innovative ideas are marching towards that goal.

Processing a material to mimic tissue architecture and strength requires planning at the material composition phase, the scaffold synthesis phase, the sterilization phase, and even the cell impregnation and attachment phase. While this section provides an overview of the considerations and processes available, a thorough understanding of the architecture of the tissue of you want to create, and a grasp of the biological necessities needed for those cells, are the most critical pieces of knowledge needed when performing your own research to determine what you need to do to produce the appropriately processed biomaterial.

Polymers as Cell Scaffolds

The perfect polymer structure shaped exactly like organ tissue down to an angstrom-level of detail is completely worthless if the cell does not respond to the material. A cell has integrins, cadherins, cytoskeletons, growth factor receptors, and more components that respond to the environment around them. If the cell feels that the

polymers surrounding it do not properly engage its chemo-mechanical sensors, then that cell will respond either by changing itself or dying. Cells do have the capacity to shape their own environment and produce collagen, integrins, elastin, fibronectin, or their own growth factors to shape their environment and the surrounding cells. Unfortunately, the cell's process of adapting itself and its environment to better suit itself is often permanent, changing the cell so that it cannot re-adapt itself to a better environment and form the functional tissue which it was intended to be.

This section will address the various responses cells have to polymers and their architecture. We will address the polymer's biochemistry, mechanical force, and nano-scale features that cells can detect and adapt as a result. We will also note that every tissue has differences, and this means in different tissue engineering techniques for different cell and tissue types.

Cell Response to Polymers

"Putting lipstick on a pig" is a clever idiom that drives home an important point: it is much more difficult to make something attractive if you start with something very unattractive. While synthetic polymers such as polycaprolactone, poly-lactic acid, or almost anything with "poly" on it, is often easier and cheaper to synthesize, process, and shape, there are numerous considerations that are negative for the living cell. First, these easy-to-process polymers are often hydrophobic, which means that the cell membrane and its outward-facing hydrophilic molecules will be repelled by the polymer surface. Second, these polymers are repeating blocks of the same molecule which has little familiarity or meaning to the cells exposed to them. A cell on the surface is just as likely to see these polymers as a foreign object rather than a structure fit to become an organ.

Early scaffold designs with materials such as polycaprolactone focused on the cell's ability to penetrate a scaffold's porous network and proliferate, and for certain cells such as fibroblasts and the periosteal cells used to coating bone this was all that was needed to produce impressive results [56]. However, the addition of natural polymers such as collagen [57] or synthetic peptides nanofibers that coat hydrophobic polymers [48] were shown to improve the proliferative properties of the scaffold over bare polymer. Mesenchymal stem cells cultured in pro-chondrogenic media on electrospun nanofibers made with a blend of polycaprolactone and ECM derived from cultured MSCs expressed more pro-chondrogenic gene activity [58]. While the physical and mechanical architecture of scaffolds is critical, if the cell can't properly attach to the polymer used then the engineering is all for naught. While a strategy to improve interaction between cells and synthetic ECM involves wholesale addition of components such as collagen and elastin or heterogeneous ECM, more targeted strategies involve the functional modification of polymers with cell-responsive amino acid sequences. Laminin-derived RGD and YIGSR for cells such as cardiac muscle showed greater adhesion and contractile fiber expression [46], and elastin-derived VAPG appears to be selective for smooth muscle [51].

Cell Response to Polymer Architecture and Mechanical Properties

Building a house on a foundation of sand is a bad idea for an obvious reason: it washes away. But assuming the house is far away from the ocean, the inevitable result is that parts of the frame, or skeleton, of the house will lack a structure to rest on and shift, resulting in the movement and cracking of any wall or shelf or portrait lying on that part of the frame. The extracellular matrix of tissue is both a foundation and a frame: it serves as the foundation for cells upon which rest their cytoskeleton, and it serves as the direction and structure of the organ tissue upon which the organ's features are patterned and positioned. A solid and firm foundation for certain tissues' cells makes sense: bone is hard and firm, a liver or a pancreas is a mechanically static organs, and kidneys don't go anywhere (unless you are giving one away). However, unlike a house, many organs exist in a constant state of movement and tension: lungs and bladders expand and contract, blood vessels and neurons stretch with the movement of our body, muscles and tendons pull on our bones, and even bone responds to the muscles' site of tension by forming tubercles. Function does manifest form in a cell's response to polymer scaffolds, and with this knowledge mechanical properties and architecture is being engineered into new polymer designs not just to mimic the target organ tissue, but because the cells require it for proper development.

Fibers are emphasized in tissue engineering because the collagen and elastin proteins that cells reside on are fibers. The evolutionary reason for this is likely out of convenience, as the building blocks of all life (amino acids, nucleic acids, carbohydrates) arrange themselves as either single or branched chain polymers. Architecturally, fibers are the logical scaffold component because they provide physical directional cues, size differences, mechanical force direction, and differences in density of packing for porosity. Cells have biochemical means (known or apparent) to detect or affect each of those parameters.

Architecture

Fiber arrangement is a critical cue for cell growth patterns. We know that mesenchymal cells [59, 60], neurons [29, 61], smooth muscle [62], even breast cancer cells [63], and likely other cells types grow along an axis cued by the substrate they grow upon. Cells which form solid tissue possess integrins, membrane-bound composed of two subunits (α and β) that bind to particular amino acid sequences found on the ECM components. Integrins are biochemically linked to a number of cell signaling pathways, but physically they are attached to α-actinin or talin and/or vinculin, which then links it to the cell's thin filament actin which is a critical regulator of cell shape. This serves as a biochemical and physical link to the scaffold on the outside [64], and cell with mutations causing deficits in the binding capabilities between actin and vinculin demonstrate cell spreading deficits [65].

Cells not only grow and align their cytoskeleton in the direction they are pointed, but they also express genes and produce proteins because of their alignment

(or lack thereof). For neurons, fiber alignment increases the expression of a myelin-specific gene for the neural-insulating Schwann cells [66] and increases pro-neuron gene expression in stem cells [50]. For bone tissue engineering, aligned fibers increases cell-to-cell junctions [67] and osteogenic markers for mineralization [67, 68]. Endothelial cells, which form the inner walls of blood vessels and capillaries, increase the expression of cell-to-cell connecting cadherins and are less likely to detach under fluid shearing [69]. It is important to note that the cells which benefit from fiber alignment are cells that require alignment of some sort. Using grooved culture plates, it was shown that fibroblasts are more responsive in their growth to designed culture templates than epithelial cells [70]. This is sensible because epithelial tissue like skin, lung, and GI are regularly enduring forces in all directions. While most tissue (such as bone) will receive force perpendicular to their normal axis of alignment, those tissue often rely on inherent material properties to bend and not break. Bone, muscle, and vessels are designed for pulling and shearing forces in a particular direction, and their axis of orientation is sensible for that purpose therefore their cellular biochemistry to respond to directionality.

Fiber diameter is also an important component to tissue engineered architecture which cells respond to. Collagen starts as a trimer tropocollagen fibril 1.5 nm in diameter, but bundles into fibrils with diameters from 10 to 500 nm apparent under SEM, and further bundles into macroscopic fibers up to 100 μm in diameter [71]. How the cell responds to fiber diameter depends on the cell type, but the size of collagen fiber bundles give us a hint as to what the threshold is. Endothelial cells cultured on fibers with diameters of 300 and 1,200 nm proliferated more than endothelial cells cultured on fiber mats greater than 7 μm [72]. Bone marrow derived mesenchymal cells expressed more markers for several ECM materials when cultured on 300 nm diameter fibers compares to 2.3 μm diameter fibers [60]. Smaller does seem to be better, and this would correlate with the diameter of collagen fibrils, but an exception seems to be neurite extensions from neurons, which did not extend as far on 300 nm diameter fibers compared to 800+ nm diameter fibers [73]. This leaves plenty of room for theorizing as to what a cell is looking for in its foundation, but it is likely different for every cell and tissue type, and some logical inferences will likely help in this exploration. For cells that are looking to cover area, such as epithelial and endothelial cells, their basement membranes and densely packed and impenetrable (unless those cells become cancerous and metastatic). For cells like neurons reaching around and between tissue to find organ, the topology and architecture for their scaffolds will likely serve that purpose better. And as new techniques for scaffold design create smaller details, a critical eye towards how the material or its breakdown products could potentially mimic irritant ultrafine particulate matter (<100 nm) and upset immune modulators should be considered [74].

Mechanical Properties

We use the mechano-sensitive biochemistry of cells daily. Although our brain interprets mechanical properties through special force-sensitive neurons, individual muscle cells also detect tension and will grow or atrophy in response. Non-muscle

cells do their own force and tension sensing, and respond to the firmness or softness of their substrate by the mechanical forces transduced through ECM-binding integrins to the internal proteins such as talin, vinculin, paxillin, actin and myosin [75]. Tension through these proteins result in phosphorylation of these proteins and activation of downstream pathways [76, 77]. The degree to which cells can sense mechanical resistance can be quite sensitive: mesenchymal stem cells will spread out and flatten over a firm surface (as determined by cytoskeletal actin staining), but place a soft gel over the surface and the degree to which the cell stays compact and rounded correlates with the thickness of the gel with a sensitivity of approximately 20 μm before the cell struggles to sense the hard surface below [78]. Spreading is fine for cells the need to cover a surface or an injury, but cardiac myocytes demonstrate that they have better organized contractile fibers and more calcium stores for contraction [79], although there does appear to be a threshold for the degree of stiffness which best functionally controls cardiac myocytes [80]. Bone remodeling is an example of cells that respond to create their environment, proliferating on hard surfaces, and mineralizing softer material [81].

When testing these properties in designed scaffolds, do take note that varying one property can affect the other. Fiber diameter, polymer types, and additives can affect the mechanical properties such as strength and density of the scaffold, and it can be difficult to tease out which of these is the cause of the phenotype change in the cell [72]. These measurement techniques are outlined in Sect. “Polymer material properties and functional tissue replacement”.

Hidden Cell-Modulating Molecules in Polymers

Before polymer customization and scaffold design was an important part of what is now called tissue engineering, biologists and physiologists were trying to elucidate the molecules animal tissue needed to grow. The original tissue engineering was determining what to coat a dish with for the cells to attach and to add extracts of blood for the proper growth factors. Diligent research developed pre-treated plastics for attachment and an assortment of growth mediums specific for cell types. Standards for tissue culture techniques allowed for experimentation with the substrates these cells were grown upon. As the field matures and tissue engineering combines synthetic processes with natural processes and polymers, we are discovering that our own native polymers have growth factors of their own, separate from the traditional soluble factors.

Collagen is a significant component of the extracellular matrix, and breakdown of the ECM inevitably releases collagen fragments. These fragments could have implications that should be considered when designing a polymer scaffold with these components. Collagen 6A3 when expressed leads to the loss of contact-inhibited growth [82], and soluble collagen 1 encourages pancreatic cancer spread and migration in a similar fashion to pro-cancer myofibroblasts [83]. Laminin χ2 chain, a component of a cell-adhesive ligand for integrins important in modulating

cell attachment and migration, can also activate the epidermal growth factor receptor when processed and solubilized [84, 85]. Hyaluronan can also be released from ECM breakdown; hyaluronan serve as a potent immune signaling molecule through TLR4 [86], and as a pro-fibrotic agent via CD44 on the cell surface [87].

The inherent cell-modulating peptides and molecules in a polymer scaffold can also mimic the pathology they come from. Whole-lung of mice treated with pro-fibrotic agents and then homogenized and used as a coating over synthetic polymer nanofibers induced pro-fibrotic gene expression in bone marrow-derived cells [88]. This research does not specify whether the causative agent in the lung homogenate is trapped soluble factors or a change in the composition of the ECM, but it does highlight that not all ECM is alike, even between the same organ. Changes in the ECM's composition affect the way cells respond.

It is very difficult to control for every possible circumstance in material, synthetic or naturally-derived. It is important to be aware of what can happen, and consider the possibilities if cells do not respond to an environment as expected. Considerations for polymer effects need not be limited to influencing the cells they are designed to contain. For example, the inclusion of polyphenols into polymer coatings scavenge free radicals and prevent immune destruction of implanted cells such as sensitive pancreatic beta islet cells [89]. Having a strong knowledge of the biology of your scaffold, your cell, and any factor that can interact with it will help lead to an effective and medically feasible design.

Polymers as Cell Scaffolds Beyond Regenerative Medicine

As scientific knowledge advances, we are running out of simple solutions to serious diseases and problems. Most research into the physiology of a disease state relies on a model to experiment upon without actually running sacrificial experiments on ailing patients. Where a tissue culture doesn't quite meet our needs, a mouse or larger mammal might suit the purposes (with some ethical questions and serious monetary costs) but at the expense of losing the control of a culture and the subtle differences between primate and rodent biology. This is where polymers as cell scaffolds and tissue engineering techniques can make an immediate impact in medicine before the regulations and hurdles of product development are met and crossed for human medical consumption. Human tissue engineered in realistic models can provide the native response of our own species in a life-like environment. Short of a whole human, these types of models can give a more realistic picture of cell-level human physiology. The three-dimensional design of scaffolds better allows for determining how tumor cell-to-matrix interactions work compared to tissue culture [90]. Scaffolds with depth and thickness provide a better model for angiogenesis [91], a potentially powerful target in many solid tumor treatments. Like any other cell, cancer responds to mechanical cues, both tension [92] and fiber direction [63], which serves as another potential target considering. While it is important to use appropriate cell types specific for the pathology being investigated,

the use of fibrotic lung ECM with a tissue-engineered polymer to induce fibrotic characteristics in mesenchymal cells [88] is a potential model for pulmonary fibrosis that allows for a degree of control that is difficult to match in a mouse model.

As with any research, the way you design and control your experiment will determine the insight you can get. With polymer scaffold design, you create avenues of research that can be controlled far better than live models, yet can provide a closer picture to reality than by remaining inside a glass dish. Many advances in tissue engineering will provide the most benefit to human health by providing a more-accurate in vitro testing platform.

Polymers as Drug or Growth Factor Delivery for Tissue Regeneration

Polymers have a dynamic range of applications and are essential to the design and innovation of the next generation of drug delivery, release, and targeting systems. Polymers as drug delivery systems exploit the capability to tailor their properties by modifying the components, method of assembly, or mechanism of release. These are important concepts such as the physical and material properties, solubility, biodegradability, drug release kinetics, and ability to sense and respond to environmental conditions/stimuli. Some of the main polymer systems used in drug delivery include polymeric micelles for drug delivery, dendrimers, hydrogels, and other scaffolds or implants. This section will discuss important concepts in design of polymers for drug delivery, specific polymer drug delivery systems, and approaches for drug delivery to clinical targets in tissue regeneration.

Important Concepts in Drug Delivery in Tissue Engineering

The *physical* and *mechanical properties* of drug delivery systems are essential in matching the needs of the target. For example, one useful property of macromolecules used in drug delivery for cancer is that they have the passive ability to accumulate in tumors due to increased accumulation of serum due to leaky vasculature in the tumor and decreased lymphatic drainage [93]. Other important physical properties include: ability to mimic the tissue stiffness, implants without corners, carriers small enough to cross membranes, or even large enough to keep them out of certain tissues.

Biodegradability and compatibility are important considerations for drug delivery system. For a polymer to be *biodegradable* it requires there to be hydrolytically or proteolytically breakable bonds in the backbone or as a cross linker. Degradation reactions are often non-linear because the degradation products of the polymer are acidic and catalyze an increase in degradation [94]. One major advantage of polymer bound drug conjugates is their increased blood circulation time resulting from their ability to escape filtration by the kidney. The potential downfall of escaping kidney

filtration is over exposure of the drug to the body. However, carriers are often biodegradable in serum conditions, and the time before the polymer is broken down can be controlled through its design. Drug delivery vehicles in solution or as an implant need to be easily broken down to prevent accumulation and a chronic inflammatory response [95]. If the vehicle is non-toxic and degradable, new tissue will readily heal. However if the vehicle is non-degradable or inert, it could be contained in a fibrous capsule by the body [95]. Biodegradability can also be used as a mechanism for controlling drug release over time, for example systems using poly lactic acid (PLA) and poly lactic co-glycolic acid (PLGA) allow the designer to determine the degradation time based on the ratio of lactide and glycolide polymer components [96]. An additional consideration when designing biodegradable polymers is that the degradation products should be non-toxic and small enough to be cleared through natural mechanisms. Products that are not cleared could build up in tissues and cause toxic or inflammatory reactions [94]. Toxicity assessment is detailed in Sect. "Polymer material properties and functional tissue replacement".

Control over the release kinetics of drugs from polymer drug delivery systems is paramount in design. Regulation of drug release is the most important separation from traditional drug delivery in which the drug levels in the blood or tissue increases during administration, peak, and then decline rapidly making it difficult to navigate between toxic or ineffective levels of the drug in vivo [97]. Release kinetics can be controlled by the system used sequester the drug, the polymer components, the amount of drug in the delivery vehicle, and environmental conditions. Controlled release systems improve the effectiveness of delivery by modifying the release profile of the drug, ability to cross biological barriers, bio distribution, clearance, and stability [98]. This level of control is especially useful when the natural distribution of the drug would cause side effect by interaction with non-target tissues and if normal administration does not allow the drug to reach its site of activity due to degradation [98].

An especially exciting research thrust is in the area of responsive or externally regulated delivery systems in which the polymer system containing the drug can be manipulated to increase release, pulse release, or stop release in response to a stimulus. Systems using this strategy are increasingly being referred to as smart scaffolds. Stimuli include temperature, pH, magnetic modulation, ultrasonic waves, or electrical stimulation [7, 9–12]. Smart polymers could provide unique solutions to old some of the most difficult challenges such as cancer drug delivery, specific tissue targeting, and direct control drug release [99].

Polymer Delivery Systems

Polymeric Micelles

Polymeric micelles are made up of hydrophilic and hydrophobic block copolymers in the form of a sphere in which the hydrophobic portions form the core and the hydrophilic regions form the shell. The hydrophobic structures in the middle of the

micelles act as a reservoir for drugs, proteins, or DNA while the hydrophilic shell acts as an interface with the biologic environment. This allows for the solubility of otherwise non-soluble payloads [100].

Micelle-forming polymer drug conjugates are used to directly incorporate and stabilize a drug onto the polymer. The drug is attached to functional groups on the polymer backbone by hydrolysable chemical bonds. Depending on the number and location of functional groups single or multiple drug molecules can be attached to each micellar unit [100].

Instead of chemical attachment, drugs or growth factors can be sequestered in micellar nano-containers through hydrophobic interactions or hydrogen bonds with the polymer backbone. Several methods of encapsulation are used to incorporate drugs into micelle nano-containers. In the *dialysis method* the block polymers and drug are dissolved in an organic solvent and dialysis against water is used to gradually replace the organic solvent with water. The replacement with water causes the self-association of the block polymers resulting in the encapsulation of the drug within the micelle core. Non-loaded drug is removed through the dialysis bag while the drug loaded micelles are trapped inside [100, 101]. The *oil/water emulsion method* uses a selective solvent for the core polymers (hydrophobic) of pre-prepared micelles, and then drug dissolved in organic solvent is added to the micelle solution with agitation. The organic solvent is evaporated from the solution resulting in the encapsulation of the drug in the micelles [100]. The *solvent evaporation method* is performed by combining the drug and polymer in a volatile organic solvent followed by the complete evaporation of the solvent and resulting in a polymer and drug film. This and aqueous phase is then added to the film and agitated resulting in the encapsulation of the drug. While this method has scale-up advantage it can only be used in conjunction with block-polymer films that have high hydrophilic lipophilic balance values so it can be readily reconstituted in aqueous solution [100, 101]. The *co-solvent evaporation method* uses a volatile, water miscible, organic solvent to dissolve the drug and polymer. Encapsulation through self-assembly is caused by the addition of an aqueous phase followed by evaporation of the organic phase [100]. The final method for forming micelle nano-containers is the *freeze drying method*. This utilizes a freeze dryable organic solvent to dissolve the polymer and drug components, and then the solution is mixed with water and then freeze dried. Isotonic aqueous solution is added to the freeze dried product to create the drug encapsulated micelles. This method is useful for scale up, however the insolubility of some block polymers in freeze dryable organic solvent limits it uses [100].

Therapeutic drug molecules that carry charge can be delivered using polyion complex molecules in which drugs are incorporated into the micelle through electrostatic charge interactions between polymers and oppositely charged drugs/molecule. The association of the drug with the core-forming block polymer cause the self-assembly of polyion complex micelle [100].

Polymeric micelles have the potential to be useful for drug delivery as they can be tailored to specify biological destination, increase specificity to a certain organ/tissue, or make them responsive to a certain condition or stimulus [100]. Drug can

be released from copolymer-drug conjugate micelles via two mechanisms; micellar disassociation and drug cleavage or water penetration into the micelle followed by drug diffusion form the micelle. Drug release from micellar nano-containers occurs solely through diffusion from the hydrophobic core, while polyion complex micelles release drug molecules though ion exchange. Polymeric Micelles are useful because they allow solubilization of hydrophobic drugs which allow them to deliver some of the most challenging molecules [102]. The relatively small size of the complexes, stability, and ability to be tailored for drug of choice make polymer micelles effective for drug delivery [100, 102].

Dendrimers

Dendrimers are a growing class of macromolecules which are known for their highly branched architecture and extensive surface functionality. Dendrimers are complex molecules and have a set architecture consisting of an interior core where branching will begin, and interior layer including several generations of repeating branched polymer units, and exterior layer attached to the outermost branched generations. These structures are often created by well-controlled reaction steps that contribute new molecules onto each layer resulting in a globular or spherical shape with 1–20 nm as the diameter range [103, 104]. Dendrimers benefit from unique properties including uniform size, extensive branching, water solubility, and multivalency that make them interesting prospects for many drug delivery applications.

Classically, there are two main methods for synthesizing dendrimers, the *divergent method*: growth originates from a core site and perpetuates radially branch by branch, and the *convergent growth process*: several dendrons are reacted with a multifunctional core to obtain the final dendrimer [103]. The divergent growth method begins with a core which is reacted with protecting branching sites. The protected groups are then removed and the free active sites are ready to react with an additional layer of branched polymers. The reaction is iterated until the desired size (branching) is obtained. The divergent method requires extensive monomer loading and chromatographic separation however is preferred method of many large scale producers. If the divergent growth method could be described as “inward-out”, conversely the convergent growth process could be described as “outward-in”. Convergent growth starts with what will become the outermost layer units and systematically works in by linking outermost units with monomers. Once optimal dendrimer size is obtained the massive branches are attached to a common core molecule resulting in a complete dendrimer [103]. The convergent growth process has the benefits of minimizing side reactions and the ability to control precise molecular weight and specific functional groups locations, as well as being easier to purify in the early stages. However this method is limited to production of lower order dendrimers and suffers from low yield when synthesizing larger structures [103].

Additional approaches have been developed and studied that build upon the major methods and address their disadvantages. The Hypercores and Branched

Monomers' growth method focuses on the speed of the dendrimer reactions. This method uses the pre-assembly of oligomeric units which can be combined to give dendrimers in fewer steps and/or higher yields. The Double exponential growth method allows for both divergent and convergent growth from a single starting material. The products are convergent and divergent trimers protected and repeated again for exponential growth. *Lego chemistry* uses highly functionalized cores and branched monomers to prepare phosphorus dendrimers. The method allows multiplication of terminal surface groups from 48 to 250 in just one step requiring minimum volume of solvent, easy purification, and environmentally non-hazardous byproducts. *Click chemistry* allows for dendrimers with multiple surface groups to be obtained with high purity and excellent yield [103, 104].

Dendrimers have been celebrated for their potential as a drug delivery tool due to their highly controlled branched and functionalized architecture, water solubility, nano-size, biocompatibility, ability to control peripheral charge (polyvalency), and drug release kinetics [103–106]. Dendrimers can be immediately introduced to blood circulation and can be absorbed across various epithelial barriers, although ideally design includes specific tissue targeting to increase therapeutic effect and decrease toxicity of free drugs in non-target organs [105].

Hydrogels

Hydrogels are defined as polymeric networks with a three dimensional architecture with the ability to absorb and sequester large amounts of water or biologic medium due to the presence of hydrophilic groups [107]. The hydrogel network can be hydrated to varying degrees depending on the environment and polymer composition. Commonly, hydrogels exhibit physical properties similar to actual tissues including low interfacial tension between the sequestered fluids and their environment. Additionally, instead of dissolving in an aqueous environment hydrogels tend to show a swelling behavior resulting from crosslinking in the structure. Crosslinks are classified as either physical or chemical and are the result of colvalent bonds, hydrogen bonds, van der Waals interactions, and physical entanglements [107]. Hydrogels can often be readily tailored to control mechanical properties, release kinetics, and degradation rate. Additionally, a class of "smart" hydrogels can be designed to respond to environmental cues to increase or decrease drug release through a specific, physical or chemical, stimuli/gradient [107]. Modern hydrogel research with respect to biologic use first started in 1960 and in the last decade the number of publications discussing the topic has increased exponentially. Recent advances and interest in using "smart" polymer systems is also driving research in the field [108].

Hydrogel use hinges on the polymers used in the system as well the the technique for formation and cross linking. *Chemical crosslinking* is effective at making mechanically stable hydrogels, the chemical crosslinking agents used in the process are often toxic and could have unwanted reactions with the bioactive agents in the gel [109]. Chemical cross linking methods include cross linking by radical

polymerization, chemical reaction of complementary groups, with aldehydes, with addition reactions, using condensation reactions, using high energy radiation, and using enzymes [109]. *Physical cross-linking* methods are growing in popularity because they don't require chemical agents that would need to be removed or that could potentially damage the delivered drug or substance. Physical cross linking methods include cross linking by ionic reactions, crosslinking by crystallization, using block and graft copolymers, hydrogen bonds, and protein interactions [109].

Some hydrogels have the ability to *self-assemble* under certain conditions, such as specific temperature and pH conditions. Injectable co-polymer hydrogels with this ability can be used to encapsulate drugs or cells for treatment of otherwise difficult to reach locations by injecting the solution to the site of action and then allowing the gel to form at body temperature and pH [110, 111]. Some polymer complexes that can be used in this capacity include PEG/polyester copolymers, polyphosphazenes, polypeptides, chitosan among others [112, 113].

In addition to synthetic injectable hydrogels, extracellular matrix hydrogels have been derived from decellularized tissues such as cardiac, dermis, adipose, bladder, and lungs [114–117]. These hydrogels are processed from the isolated extracellular matrix scaffold remaining after tissue decellularization usually including the natural polymer and proteins that make up every tissue. These scaffolds are also thought to sequester some of the naturally bound growth factors from the specific tissue type allowing them to be particularly bioactive in a regenerative role. Naturally derived ECM hydrogels benefit from an more compatible interface with the tissue, the ability to form gels at physiological conditions for inject ability, and has pro-regenerative degradation productions [118, 119]. In addition to delivering growth factors these gels have been used to deliver cells and other drugs through encapsulation.

Implants for Tissue Regeneration

In addition to standalone drug delivery systems such as hydrogels, polymeric micelles, and dendrimers whose main focus is drug delivery, polymers can be incorporated into larger implants or scaffolds and drug delivery may even be a secondary mechanism of the system. An example of this is a joint implant with a polymeric coating that elutes inflammation reducing drugs to help with healing/rejection, or an electrospun vascular graft that has bound growth factors to stimulate endothelial growth or angiogenesis [120]. Drugs can be coated onto polymers for direct dilution from of the surface, bound to functional groups on the polymers, or sequestered in the polymer structure itself. Drug eluting scaffolds can be used effectively for long term.

One class of scaffolds that can be used as a surface or implantable drug delivery scaffold system are created using *electrospinning* technique. This process, as described in Sect. "Polymer processing techniques to mimic tissue architecture and strength" can be used to create porous nanofiber scaffold from a variety of polymer substrates and has the potential to be combined with therapeutic drugs for a sustained release of the molecule when the scaffold is implanted in vivo [121]. The process can

be controlled to create scaffolds with a range of fiber sizes, orientation, and porosity to effect drug release profile [121]. Electrospinning for drug delivery has applications in wound healing, long term treatment of heart and vascular disorders, and cancer treatment among others [122]. Electrospinning can be done using both naturally derived and synthetic fibers, as an emerging technique in scaffold design it may overcome current limitations with drug or growth factor delivery [120, 123].

Polymer Coatings are another example of a secondary purpose drug delivery system, were they are used in conjunction with a main implant, such as a stent, joint replacement, or other permanent implant [124]. Coating like this can be used to attenuate negative interactions between the implant interface and the tissue, and even promote tissue ingrowth into the implant. Bone ingrowth into knee, hip, or other replacements is one huge application for these coatings. The main reasons that orthopaedic implants fail is because of bacterial infection, chronic inflammation, and limited bone integration with the implant [125].

Polymer Material Properties and Functional Tissue Replacement

Classical tissue engineering follows the paradigm cells + scaffold = tissue replacement. In order for tissue replacement to occur, the polymeric scaffold must degrade. There are two common degradation mechanisms of polymeric materials in the body: swelling/dissolution and chain scission. In swelling/dissolution, hydrophilic domains of the material swell and dissolve in the body. In the chain scission, primary bonds of the polymer are broken through hydrolysis or oxidation. Polymeric properties that influence hydrolysis are the reactivity of groups in polymer backbone, extent of inter-chain bonding, and amount of water present. In chain scission by oxidation, reactive oxygen species attack and break covalent bonds. The three steps to oxidative chain scission are initiation, propagation, and termination. The extent of oxidation depends upon the number of susceptible domains in the polymer. Typically, lower molecular weight polymers and those that are not heavily crosslinked will have faster degradation due to fewer secondary and tertiary interactions. Polymeric materials for use in tissue engineering may be designed to have inherent domains for intentional hydrolysis or enzymatic degradation.

Biomaterials paradigms for designing polymers for functional tissue replacement follow criteria believed to be good for cellular ingrowth and formation of functional tissue. The criteria often followed for material properties that may be tailored for specific tissue types are 1. Polymer degradation rate 2. Polymer fiber diameter 3. Polymer pore size 4. Polymer hydrophilicty/hydrophobicity 5. Polymer mechanical properties (often influenced by numbers 1–4). Discussion of each of these criteria in specific tissue engineering examples and techniques for measurement follows.

Polymer Degradation Rate

Depending on the tissue to be engineered, and the approach chosen for processing the polymer, the degradation rate may be the most important factor when choosing a polymer for tissue engineering. One goal of classical tissue engineering is for the scaffold to degrade as it is replaced with regenerated tissue [126]. For example, if you are engineering a functional tissue replacement for skin, you may want to choose a polymer or polymer combination whose degradation rate will match the typical wound healing timeframe for proper tissue influx [127]. When properly designed, polymer scaffold degradation may be associated with remodeling of the collagen network in engineered polycaprolactone bisurea (PCL-U4U) thermoplastic elastomer scaffolds by human saphenous vein vascular derived cells [128]. In these PCL-U4U scaffolds, the vascular derived cells followed the orientation of the remodeled fiber architecture. For tissue engineering architecture that you do not expect to be replaced by the patient's own tissue, such as a heart valve or dental implant, you will want to choose a polymer with little to no biodegradation. The stability of the polymer in vivo is an incredibly important variable to ensure long term effectiveness of the engineered tissue.

Polymer degradation rate may be measured in vitro or in vivo, with in vivo testing being the most reliable. A typical degradation test in vitro would involve first obtaining an accurate weight of the engineered polymer. Next, the engineered polymer tissue is placed in a saline bath at 37 °C. Samples may be taken from the saline bath over time and analyzed for the polymer concentration in the bath using mass spectrometry or other chemical analysis. At the end of a set period, the polymer will be taken out of the bath and a final weight will be obtained. This in vitro technique has limitations. The first limitation is that the simple degradation measurement does not take into account any cellular or metabolic activity. The second limitation is that the weight analysis does not take into account any swelling or increase in water weight of the polymer. Another in vitro technique that may be used is similar degradation measurements with the addition of cells + media. However, this technique cannot replicate the complex in vivo environment. The most common in vivo degradation test is to perform a subcutaneous implant of the polymeric material in the side flank of a mouse. Mice may be euthanized at various time points and the area may be assessed histologically for degradation of the polymeric material. This in vivo testing will provide basic information about how an immune response and tissue environment may affect polymer degradation. However, this side flank model may not be adequate in assessing the degradation of polymers used to engineer tissues that undergo large or repeated mechanical loads. The side flank model is often a needed first step towards assessing polymer degradation. Further degradation testing is usually necessary in vivo in models that mimic the disease or injury for which a functional tissue replacement is needed.

Polymer Fiber Diameter

Polymeric fiber diameter is important for several reasons in tissue engineering, some of which are discussed earlier in this chapter. The fiber diameter will play a large role in both the cellular infiltration into the scaffold and the mechanical properties of the scaffold. In order to engineer functional tissue replacements for structures that have high amounts of fibrillar collagen, the polymeric fiber diameter is increasingly important. Such tissue structures include tendon, heart valves, the urinary bladder, vascular grafts, or ligaments among others. These are typically tissues that undergo large changes in mechanical forces and must have the structural fortitude for these load bearing situations. Fiber diameter may also play a role in the immune or foreign body response of cells to the polymeric scaffold. One study showed that fiber diameter in the nanometer scale caused a pro-regeneration response of macrophages, while a larger fiber diameter in the micrometer scale caused a pro-inflammatory response of macrophages to the poly-l lactic acid (PLLA) scaffolds [129].

Polymer fiber diameter will depend upon the type of polymer or polymer blend used and the technique of polymer processing. These techniques are outlined in Sect. "Polymer processing techniques to mimic tissue architecture and strength". Polymer fiber diameters are most adequately measured with imaging based technology. For example, in a poly-l lactic acid (PLLA) electrospun polymer scaffold, fiber diameter is commonly measured by first performing scanning electron microscopy followed by image analysis and statistics to calculate average fiber diameter size. The image analysis technique may also work well for other polymer processing techniques such as hydrogel formations and woven scaffolds.

Polymer Pore Size

The easiest way for a cell to penetrate the scaffold material and form a three-dimensional engineered tissue is through large interconnected pores. Cells will range from 5 to 50 μm and can squeeze themselves through pores ranging from 3 to 20 μm. Many factors will influence whether or not a cell will penetrate the pore including nutrient availability, growth factor or cytokine recruitment, and cell phenotype. While these biological factors may be engineered into the polymer or cell choice, the physical factor of pore size will usually be altered through processing techniques.

The pore size measurement may be done with image analysis, similar to that described above for polymer fiber diameter. However, this imaging based technique may not be adequate for polymers with three dimensional and non-uniform pores. A measure of porosity may instead be adequate using an liquid exclusion porosimetry measurement [130]. In liquid exclusion porosimetry, the scaffold is immersed in pure ethanol or other solvent that will disperse throughout the polymeric scaffold under a non-reacting gas pressure. The differential pressure

required to displace the wetting liquid is related to pore diameter by the Washburn equation [Eq. (7.1)], which states that higher pressure is required to remove liquid from smaller pores:

$$p = 4\gamma \cos\ \theta / D \tag{7.1}$$

where p is the differential pressure across the length of the pore, D is the pore diameter, γ is the surface tension of the wetting liquid; and θ is the contact angle of the wetting liquid with the sample [130]. The volume of the liquid flowing out of the membrane is collected and weighed in an analytical balance. This volume corresponds to the flow-through pore volume within the scaffold. This technique works well for electrospun polymeric scaffolds; however, it may be replaced with simpler gravimetric analysis for other scaffold formations.

Polymer Hydrophilicty/Hydrophobicity

Polymer degradation by hydrolysis will be governed by the amount of water present in the formulation. The degradation rate is thus influenced by the hydrophobicity of the polymeric side chains and their ability to take on water. The hydrophobicity of the polymeric structure must strike a balance with the optimal hydrophobicity needed for protein adsorption and cellular attachment. In order for cells to adhere to a polymeric scaffold, the scaffold must be hydrophilic. However, many of the polymers utilized for scaffold fabrication are highly hydrophobic. Thus, surface modifications or pre-wetting the scaffold with a solvent is typically needed for successful cell adherence.

Testing for hydrophilicty/hydrophobicity is most commonly performed by calculating the contact angle of a water droplet on the surface of the material. The more hydrophobic the polymer is, the greater angle will be formed between the water droplet and the surface. For example, a material with a contact angle of 90° will be more hydrophobic compared to one with a contact angle of 30°. Contact angles may be measured using imaging and standard image analysis tools or physically measured with a goniometer.

Polymer Biomechanical Properties

The biomechanical properties of the polymer used in tissue engineering are an important factor in ensuring durability of the tissue engineered structure. The biomechanical properties must be understood at the time of implant and throughout degradation of the polymer as it is filled in with regenerated tissue. For example, if a polymer is utilized to repair a defect in a mineralized, load bearing tissue, such as bone, the tissue engineered polymer construct must be able to withstand the loads during healing. If a polymeric scaffold is used in a vascular graft repair, it must be

able to withstand the shear stress environment under pulsatile flow. A clear understanding of the mechanical profile of the material and the mechanical environment at the location of the implant is necessary for a successful engineered tissue.

Common tests for biomechanical properties include traditional uniaxial testing, biaxial testing, three-point bending, compression, or torsion tests. In all of these modes of mechanical testing, the viscoelastic (or time dependent) nature of the polymer must be assessed. Biomechanical tests need to be performed on the polymer alone, the polymer + cells, and explanted tissues from animal studies. Each of these sample groups can be compared to the biomechanical properties of the native tissue that is to be replaced. These tests are often utilized in combination with finite element analysis and computational simulations to fully understand the biomechanical properties of the engineered tissue. Many experts in the field believe the mechanical tests to be the ultimate measure of a tissue engineered polymer. However, in vivo testing in relevant animal models is likely the best measure prior to use in patients.

Other Important Considerations in the Design of Biodegradable Polymers for Functional Tissue Replacement

Polymer scaffolds of conventional tissue engineering techniques must not only degrade at a kinetic rate appropriate for tissue replacement, the scaffolds must also serve as templates to engineer the proper cellular niche for cellular differentiation into a pro-regenerative phenotype. Growth factors or other additive factors may be added to the polymer scaffold for release. These growth factors or other drugs will provide a favorable cellular microenvironment and/or may serve to recruit cells to the site for regeneration. Growth factor or drug release is measured similarly to in vitro degradation rate tests as described above. Growth factor release may be assayed in sampling buffer from the scaffold at various time points via enzyme linked immunoabsorbant assay (ELISA). Many new advances in the synthesis of polymers as degradable cellular templates are being reported daily; however, few have made it to market.

As with any polymeric material that interfaces with the body, the acute and chronic toxicity of the polymer and its degradation byproducts must be taken into consideration. The best way to examine acute and chronic toxicity is through an animal model. For example, in the rat, acute toxicity would be measured within the first 24 h of implantation. Chronic toxicity would be assessed at 90 days post implantation, which corresponds to 10 % of the animal's lifespan. Toxicity is commonly assessed by material tracking in vivo through X-ray, MRI, or other non-invasive in vivo imaging such as IVIS (Perkin Elmer). At the end of the time duration, histological analysis is performed to ensure that the material causes minimal cell death at the site of implantation. For a tissue engineered implant containing a polymeric biomaterial to be safe and effective, chronic toxicity must be eliminated.

Polymers for Engineering Complex Architectures Such as the Lung and Liver

Several different polymers are utilized in order to engineer tissues with complex tissue architectures and highly specialized cell function. The focus of this section will be on the lung and liver. Both the lung and liver are organs with high need of replacement due to fibrotic disease or other dysfunction. There is high unmet demand for donor lungs and livers. Tissue engineering strategies may either fully replace the lung or liver or may be employed in assist devices to replicate function until a donor organ is available. This section will highlight the course of research in polymeric based tissue engineering the lung and liver. The state of the art in tissue engineering these two organs has followed a similar course of development as follows: polymers for structured cellular transplantation, the use of combined synthetic and naturally-derived polymer approaches to large structures, the use of decellularized tissues, and the use of polymers in assist devices.

Polymer Sponges or Hydrogels for Structured Cellular Transplantation

The basic paradigm for engineering polymeric structures for cellular transplant in the lung and liver is to utilize a hydrogel or sponge structure with either adult differentiated cells or progenitor cells. In both the lung and the liver, these polymer-cell structures have shown great success in vitro. Additionally, in vivo animal studies show promising results of cellular survival and functional differentiation.

Although the liver has some regenerative ability, there is a large need for liver transplantation due to cirrhosis, drug toxicity, or congenital defect. Tissue engineering functional liver units has been a focus for many years as a potential source for liver replacement. The first examples of functional transplantable liver-like structures used prevascularized, non-degradable polyvinyl alcohol sponges to accommodate transplanted hep-atocytes with limited success [131, 132]. Modified gelatin sponges with lactobionic acid (MGLA) have also been used to support mouse hepatocyte growth [133]. Microhydrogels made of fibrinogen attached to poly(ethylene glycol) (PEG)-diacrylate side chains were used as a cell carrier for intravascular transplantation of hepatocytes in a rat model [134]. Another naturally derived material, silk fibroin, has been utilized in several tissue engineering approaches. Silk fibroin is a naturally-derived material which has high molecular weight organic polymers characterized by repetitive hydrophobic and hydrophilic peptide sequences. Silk fibroin assembles into regular structures during materials formation and can be considered as nature's equivalent to synthetic block copolymers [135]. Due to its polymeric nature, silk fibroin has been utilized in a cryogel to support hepatocyte growth with aims for future transplantation [136]. A cryogel is a porous hydrogel formed from polymerization under low temperature

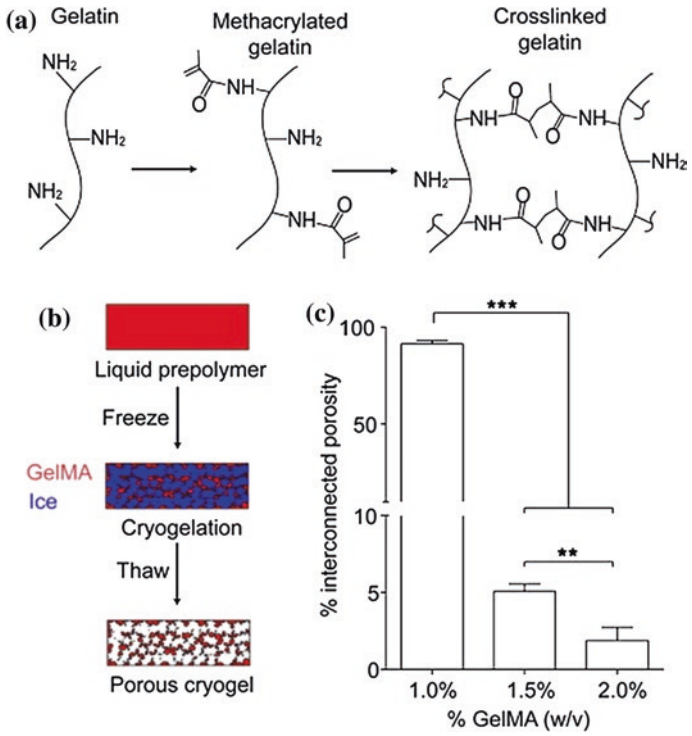

Fig. 7.1 Preparation and assessment of gelatin cryogel. **a** Schematic of GelMA synthesis and crosslinking. Pendant methacrylate groups are added primarily to the free amines of gelatin by reaction with methacrylic anhydride. Free radical polymerization results in crosslink formation between methacrylate groups. **b** Cryopolymerization of methacrylated gelatin. Freezing of methacrylated gelatin in the presence of radical initiators (APS and TEMED) allows polymerization to occur in the partially frozen state (cryopolymerization). Ice crystals formed during the freezing process and thawing after cryopolymerization results in the formation of a hydrogel with micron-scale pores. **c** Volume of interconnected pores in gelatin cryogels (normalized to total gel volume). Values represent mean and standard deviation ($n = 10$). Data were compared using ANOVA with Bonferroni's post-hoc test (**$p < 0.01$, ***$p < 0.001$). Reproduced from [163]

conditions. The ice crystals that form allow for highly interconnected pores within the hydrogel. Preparation and assessment of a gelatin hydrogel with methacrylated crosslinking is shown in Fig. 7.1.

In the lung, similar approaches have been employed focusing on naturally-derived polymers alone or in combination with synthetic scaffolds. Gelfoam sponges are commercially available surgical devices that are derived from porcine skin gelatin (Pfizer.com). Gelfoam sponges are water-insoluble and capable of absorbing up to 45 times their weight (Pfizer). Gelfoam sponges have been utilized to grow lung organotypic structures in vitro [137, 138]. Additionally, Gelfoam sponges seeded with fetal rat lung cells were viable in the adult animal lung for up to 35 days with neovascularization apparent [139]. Other hydrogels with natural

and synthetic components have been used to engineer pulmonary cell structures. The natural components of these are typically either gelatin or Matrigel. Gelatin is a low-cost, non-immunogenic natural material derived from collagen. Matrigel is a product from Corning Life Sciences that is a gelatinous extracellular matrix mixture made from mouse sarcoma cell secreted matrix. These two naturally-derived materials have been used in vitro and in vivo animal models extensively. Additionally, gelatin has been used in numerous human applications, which makes it an appealing polymer for new tissue engineered products. Gelatin in the form of a three-dimensional microbubble scaffold was used to provide the proper cellular microenvironment for differentiation of mouse pulmonary stem cells into alveolar pneumocytes [140]. Similarly, hydroxyethyl methacrylate-alginate-gelatin (HAG) hydrogels have been employed as three-dimensional structures for lung epithelial cell growth [141]. Additionally, three-dimensional structures for alveolar cell growth using matrigel hydrogel and synthetic polymer scaffolds of poly-lactic-co-glycolic acid (PLGA) and poly-L-lactic-acid (PLLA) fabricated into porous foams and nanofibrous matrices have been used successfully in vitro [142]. Engineering the cellular microenvironment for progenitor cell differentiation will likely continue to be the focus of new polymer formulations in lung and liver cell delivery.

Naturally-Derived and Synthetic Polymer Strategies for Larger Pulmonary and Hepatic Structures

While hydrogels or sponges have been examined for cell transplant for the parenchyma of the liver or lung, more traditional tissue engineering approaches have been utilized for some of the larger structures such as bile ducts, trachea, or bronchioles. These traditional tissue engineering approaches utilize scaffold materials made from naturally-derived and/or synthetic polymers and have been implanted with some success in vivo in both humans and in animal models.

In the liver, there have been few attempts to re-create functional liver structures using polymeric biomaterials in the context of engineering the bile duct or liver capsule. Three dimensional stacked polycarbonate membranes with hepatocytes showed functional liver units in vitro [143]. Functional liver units were also observed using micropatterned Poly(ethylene glycol)-poly(DL-lactide) and lactosylated poly(DL-lactide) electrospun fibrous mats [144]. For larger structures, like the bile duct or vascular structures, polymers may be formed into sheets and then tube structures. The bile duct was successfully replaced in an animal study using polycaprolactone and polylactic acid reinforced with polyglycolic acid fibers in a tube structure [145]. Continuing advances in synthetic polymer processing, such as three-dimensional printing and micropatterning, will improve the field of engineering larger liver structures such as the bile duct.

In the lung, tissue engineering strategies have been employed to engineer larger airway structures such as the trachea, main stem bronchi, and bronchiole. In 2008, the first transplantation of a tissue-engineered trachea in a human being

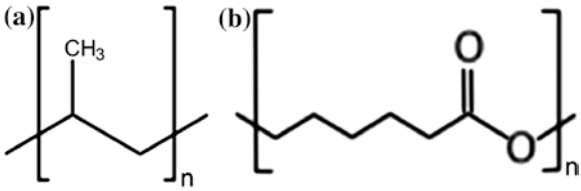

Fig. 7.2 Chemical formulas for **a** Polypropylene and **b** Polycaprolactone

was done to replace an end-staged left main bronchus with malacia in a 30-year-old woman. The implanted trachea was engineered from a decellularized cadaveric trachea seeded with autologous epithelial cells and mesenchymal stem cell-derived chondrocytes [146]. Five years post implantation, the tissue engineered trachea remained viable and patent with stenting needed in the native trachea near the implant [147]. This initial success advanced the field of clinical applications of tissue engineered airways (Fig. 7.2).

New advances and improvements on tissue engineered trachea involve synthetic and natural combination polymer strategies. Polypropylene meshes with collagen and/or poly(L-lactic-acid-co-e-caprolactone) coating have been used in an animal model for replacement of the left main stem bronchi [148, 149]. Greater success was achieved utilizing the poly (L-lactic-acid-co-e-caprolactone) coating for epithelial regeneration. Other success has been reported in a rabbit model where tissue engineered tracheas were formed from articular cartilage matrix and chondrocytes [150]. New advances in 3D printing polymers have been harnessed for applications to the airway. A half-pipe polycaprolactone 3D printed trachea seeded with mesenchymal stromal cells in a fibrin matrix was implanted into a rabbit model with initial success of regenerated epithelium [151]. Tissue engineering polymer approaches for the large airways have gained momentum due to the ease of fabrication and implantation. However, smaller airways, termed bronchioles, have been attempted for in vitro understanding of small airway diseases such as asthma with the eventual goal of a functional tissue engineered lung. For the bronchiole, a type I collagen gel seeded with fibroblasts, epithelial cells, and airway smooth muscle cells was engineered utilizing a pulsatile flow bioreactor [152]. Similarly, another study cites tubular bronchiole structures engineered using airway smooth muscle tubular structure collagen pulsatile flow [153]. The engineering of these smaller airway structures highlights the need for advances in bioreactor technology to go hand in hand with the advances in polymeric biomaterials. A true tissue engineered lung will likely utilize a combination approach of polymeric materials, the proper cell choices, and proper in vitro conditioning.

Vascularization and the Mechanical Environment

Vascularization is often the limiting factor in tissue engineering complex three dimensional tissues for lung and liver replacement. Both hepatocytes and pulmonary epithelium have high oxygen needs, so the design of polymeric structures must allow for a highly vascularized system. Furthermore, the blood supply is

crucial in both of these systems to maintain functionality. The liver's main function is to filter the blood coming from the digestive tract, and the lung's main function is to allow for gas exchange in the blood to occur. This functionality, while seemingly obvious to include, is non-trivial when engineering three dimensional structures for replacement of liver and lung. Several strategies have been employed to improve oxygenation of cells grown in vitro including the addition of perfluorcarbons and the bubbling of oxygen through the culture media.

In addition to the flows from the vasculature, the mechanical environment of the three-dimensional construct must be taken into account. When engineering a portal triad for the liver, the shear forces in the bile duct must also be considered in the design. Furthermore in the lung, a very complex mechanical environment exists as the lung is constantly distended and relaxed. The polymeric structures used to engineer the lung must have large elastic recoil in order to withstand this repeated deformation. Due to the complex mechanical environment and vascularization structure of the lung and liver, decellularized organs may provide the best natural polymeric scaffolding material.

Decellularized Organs

Decellularized organs from human cadavers or animal sources provide the structural architecture necessary for complex three dimensional tissues. Key natural polymers, proteins, and structural components remaining in decellularized organs are collagen types I–IV, elastin, fibronectin, and laminin. Depending on the mode of decellularization (as described in Sect. "Biologically Generated Polymers"), many active growth factors and cytokines may also be present in the matrix.

Lung decellularization has had successes in animal implantation [154]. Several other studies have examined effects of decellularization processes on matrix architecture and strength [155, 156]. Lung decellularization leaves the complex architecture of the lung airways and alveoli with the vascular network available for repopulation with endothelial cells (Fig. 7.3). Repopulation of decellularized lung matrix with progenitor cells and subsequent differentiation into lung phenotypes shows that the decellularized lung matrix provides a hospitable environment for regeneration [157]. Although there has been much progress in the field of decellularized lung scaffolds, it remains unclear what the proper cellular choices are for recapitulating proper lung function and air-tight gas exchange.

Liver structure/function relationship is paramount in engineering hepatic units. To retain the complex structure/function relationship, decellularized livers have also been utilized for repopulation and transplantation into in vivo models [158, 159]. Using the decellularized livers as scaffolds causes the proper cellular spatial distribution that is so difficult to achieve using other polymeric scaffold fabrication techniques. As with the lung, similar issues remain to be worked out in whole organ liver tissue engineering. These issues include clotting, cell choice, cell fate, and long term functionality of the organ.

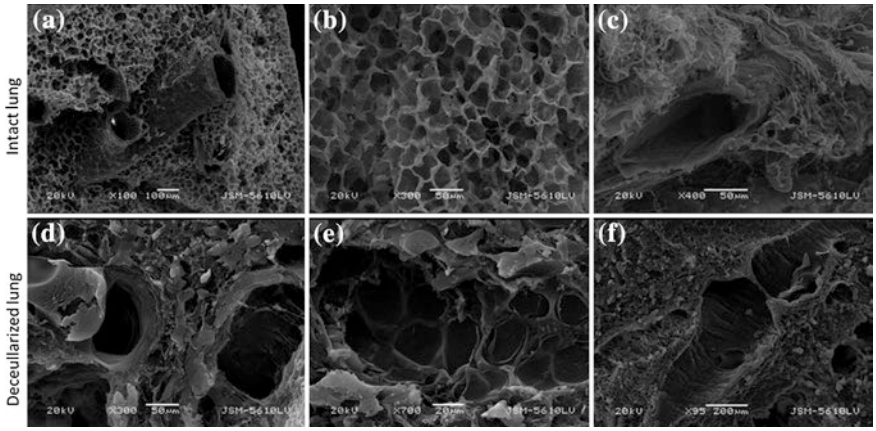

Fig. 7.3 Intact and decellularized mouse lung shows retention of pulmonary structures. **a** and **d** show larger airways, **b** and **e** show alveoli, **c** and **f** show blood vessels

Fig. 7.4 Bio-inspired 3D liver detoxification device. Polydiacetylene nanoparticles (*green*) are installed in poly(ethylene glycol) diacrylate hydrogel matrix (*grey*) with liver-mimetic 3D structure fabricated by 3D printing. The nanoparticles attract, capture and sense toxins (*red*), while the 3D matrix with modified liver lobule structure allows toxins to be trapped efficiently. This biomimetic 3D detoxifier has promising clinical application for detoxification by collecting and removing toxins. Reproduced from [164]

Assist Technologies

Due to the complexity and remaining issues in engineering replacement organs for lungs and livers, "lab on a chip" approaches have been utilized to engineer assist devices. The assist devices can provide essential replacement of function that is missing even in decellularized organs. These assist devices are meant to perform the function of the lung or liver as either a bridge to transplant or as a permanent assist technology. The majority of assist devices are fabricated using soft photolithography or three dimensional printing. In the former, polydimethysiloxane (PDMS) is typically the polymer of choice because it can be poured into a photolithographic mold. The PDMS is often coated with a naturally-derived extracellular matrix protein such as collagen or fibronecin. Cells are then placed within the channels to perform function [160, 161]. With 3D printing, the polymer choice is broadened such that any polymer that can be put into solution at a viscosity matching the printer nozzle may be utilized. For the liver, polymers have been used as the structural components of assist devices as well as to perform function by capturing toxins (Fig. 7.4).

In the lung, endothelial cells and ECM proteins have been added to polymeric tubes for enhanced gas exchange in extracorporeal membrane oxygenation devices [162]. There will continue to be a future push toward incorporating tissue engineered external devices to avoid coagulation and better prognosis for assist or hybrid technologies.

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