Scaffolds for Tissue Engineering
Scaffolds for Tissue Engineering
Biological Design, Materials, and Fabrication

edited by
Claudio Migliaresi
Antonella Motta
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Preface

Three years ago, after a conference in Italy, we received an invitation by email from Stanford Chong, Pan Stanford Publishing, to submit a book project. It was to be focused on the functional applications of polymers (the theme of the conference), or another topic of our interest.

After some months of thought, we submitted our idea, which came after long discussions between us, sometimes involving our coworkers, our students, and also scientists who we were visiting then during our travels abroad.

We were well aware of the fact that there were many excellent books on the topic, and that others will have been published before this one. However, we imagined our book as a “negotiated” synthesis between the two different approaches of tissue engineering—that of the biologist (Antonella Motta) and of the engineer (Claudio Migliaresi)—which were much more different at that time, than now.

Our common assumption was that a scaffold should be designed, in terms of materials, architecture, properties, to accomplish not only mechanical but biological requirements, and our book should properly highlight this aspect, in both title and content. This is how the title of the book, Scaffolds for Tissue Engineering: Biological Design, Materials, and Fabrication, came into being.

Often scaffolds are made by a random combination of materials, with architecture and properties that evoke unrealistic biological behaviors, ignoring the fact that a scaffold is an active device that interacts with cells in vitro and with the complex biological system in vivo. The chapters of this book, written by leading scientists in the field, many of them also good friends, reflect this idea. And upon reading the book, which we have done several times, we must state that the contributing authors have done a wonderful job.

We are grateful to them for having accepted our invitation and for the valuable time they have spent on the book. We are also grateful to Pan Stanford Publishing, which patiently forgave our delay
with respect to the scheduled times, and in particular to Stanford Chong and Shivani Sharma, who were prompt in offering help and assistance.

Claudio Migliaresi
Antonella Motta
Spring 2014
Chapter 1

Overview: An Evolving State of the Art in Tissue Engineering

Anthony T. DiBenedetto
Department of Chemical Engineering, Gant Complex, Unit 3136,
University of Connecticut, Storrs, CT 06269, USA
atdibenedetto@gmail.com

Dr. Anthony Atala of the Wake Forest Institute for Regenerative Medicine in Winston-Salem, North Carolina, encountered a young patient, Luke Masella, with a life-threatening bladder condition that required an organ transplant. Parental permission was obtained to use the patient’s own cells in an experimental procedure to create a new bladder. Healthy muscle cells taken from the patient’s diseased bladder and urothelial cells from his urinary tract were multiplied by incubation in petri dishes and then applied to a balloon-shaped scaffold made from collagen and other biocompatible organic materials. The cellularized bladder scaffold was incubated in vitro at body temperature until the cells generated functioning tissues and then successfully transplanted at Boston Children’s Hospital in a procedure that took approximately 14 hours. At this writing, Luke is a healthy, physically active student at the University of Connecticut with
the prospects of living a long and productive life. In 2003, a startup company, Tengion, was founded with an objective of advancing Dr. Atala’s basic and clinical research. A successful clinical trial of the use of a tissue-engineered organ in reconstructive surgery on seven patients with end-stage bladder disease was completed after 31 months, during which time the engineered bladders displayed normal behavior.

In 2008, a team of doctors, led by Surgeon Professor Paolo Macchiarini, of the Hospital Clinic of Barcelona, Spain, transplanted a tissue-engineered left bronchus into the windpipe of Claudio Castillo, a 30-year-old mother of two. She needed the transplant to save a lung after her air passage had been damaged by tuberculosis. To make the new bronchus, a donor windpipe was taken from a patient who had recently died. Scientists from the Politecnico di Milano, Italy, the University of Bristol, United Kingdom, and the University of Padua, Italy, assisted in the preparation of a new tissue-engineered bronchus using a portion of the harvested donor windpipe. Strong chemicals and enzymes were used to wash away all of the cells from the donor windpipe, or trachea, leaving only a decellularized tissue scaffold made of the fibrous protein collagen. Cells were taken from Ms. Castillo’s bone marrow and windpipe lining to create a tissue scaffold repopulated with Ms. Castillo’s own cells. After four days of growth in a rotating bioreactor, the newly formed donor bronchus was transplanted into Ms. Castillo. Four days after transplantation the hybrid windpipe was almost indistinguishable from adjacent normal airways. A biopsy of the site proved that the transplant had developed its own blood supply, with no signs of rejection.

Drs. Harald Ott and Doris Taylor and colleagues at the University of Minnesota harvested donor hearts from adult rats and removed all cells from the hearts by bathing them with a solution of detergent compounds. The decellularized matrix preserved the fibrous morphology and orientation of the myocardial/extracellular matrix (ECM) of the original heart, resulting in an authentic heart scaffold with blood vessels, heart valves, and an intact atrial and ventricular geometry that retained the architecture required to pump blood. They seeded these scaffolds with cardiac cells from young healthy rats and maintained them in culture conditions in a bioreactor that simulated cardiac physiology. Four days later, they observed “contractions and, by day eight, their constructs could generate pump function equivalent to about 2% of adult heart function.” After
a further period of time, both the larger cardiac vessels and the smaller third- and fourth-level branches were capable of supporting the expansions and contractions of a living heart. The techniques were also used to create “breathing rat lungs.” The work resulted in a start-up company called Miromatrix Inc. Shortly after that, a team from Massachusetts General Hospital and Harvard University used a mixture of stem cells from rats and humans to recellularize donor rat lungs and transplanted the resulting scaffolds into young rats. The lungs functioned for six hours, albeit imperfectly.3

The three above-mentioned accomplishments are examples of a myriad of research and technological advancements that are now occurring in the 21st century. The concept of an interdisciplinary field of “tissue engineering” as a unique field of study was generated during a series of panel meetings and workshops sponsored by the National Science Foundation of the United States.4 The widespread acceptance of the concept can be traced to Y. C. Fung of the University of California at San Diego, who proposed the idea at a 1988 NSF workshop held at Lake Granlibakken, California, and to Robert Langer and Joseph Vacanti, whose review article in Science promoted a rapid increase in collaborative research in the worlds of clinical medicine, developmental biology, biomaterials, biomechanics, and biomedical science and engineering.5 Studies in developmental biology of cell and tissue coalescence, cell adhesion, and the properties of embryonic tissues provided the biological foundation for modern tissue engineering. The possibility of engineering a tissue was demonstrated as early as 1930s,6,7 and fundamental studies of stress-dependent morphogenetic effects in tissue remodeling,8–10 analytical modeling of cell movement, interstitial flow and deformation in an anisotropic ECM,11 and studies of morphogenetic processes in the growth of vascular networks12 all contributed to the early development of tissue-engineered scaffolds. Since then, there has been a dramatic increase in the imaging, modeling, and simulation of human anatomy and physiology, promoted by long range strategic plans for basic research in the biological sciences and medicine, for development of computer and engineering technologies related to public health, and by the Human Genome Project.4,13–15

_Tissue Engineering_ as a field of study was defined as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole
organ.” Research on the development of two-dimensional scaffolds containing cells on sheets of naturally occurring collagen and collagen–glycosaminoglycan composites for the regeneration of new skin was a precursor to the creation of three-dimensional (3D) scaffolds capable of housing the large numbers of cells required for regenerating other organs and functional tissues. A primary objective in the creation of functional tissue-engineered scaffolds has been to design 3D scaffolds that permit the production of ECM that mimic the ECM of the tissue being replaced or repaired. To accomplish that function, one must provide a mechanically stable environment that hosts the necessary cells, growth factors, and other biological components in a porous structure that allows cell migration, adhesion and proliferation, and vascularization of the growing tissue that mirror that of the original tissue. Critical issues have included enhancing cell survival, maintaining differentiated function, developing a significant cell mass, and achieving vascularization where required. Attempts to engineer functional tissues and organs during the period 1990–1999 included scaffolds to aid nerve regeneration, corneal implants, tissue-engineered liver implants, creation of an artificial pancreas, and the development of a urinary organ using bladder cells encased in a polymer composite matrix. During this period, considerable effort also focused on creating tissue-engineered blood vessels, heart valves, and polymer scaffolds for creating heart muscle tissue.

With regard to the mechanical design of tissues, Roderic Lakes drew attention to the fact that man-made objects, in common with all living objects, including the human body, exhibit structure on more than one length scale and that structural hierarchy in large part determines bulk material properties. One of the earliest examples of the use of hierarchical design principles in architectural engineering has been attributed to Gustave Eiffel’s design of the Eiffel tower (exhibited at the International Exhibition of Paris of 1889). At a height of approximately 320 meters and a weight of 7300 metric tons it was the tallest metal structure of its time. It was designed as an open lattice, third-order hierarchical cantilevered structure in which the primary structural shape was strengthened by a lattice network of second-order and third-order girders. His hierarchical design strategy started a revolution in civil, architectural, and mechanical engineering design of skyscrapers, bridges, transportation vehicles, and other loadbearing commercial
products. In many respects, the use of computer-aided design and manufacturing (CAD/CAM) techniques that are employed for these purposes have been found to be applicable to the design of tissue-engineered scaffolding. Engineers and material scientists initially explored the application of solid mechanics, fluid mechanics, and CAD/CAM, using "top-down" reverse engineering strategies in the design of orthopedic and orthotic devices manufactured from biocompatible metal alloys, ceramics, and fiber-reinforced plastics. Total hip replacements consisting of a femoral ball that fits into the socket of the hip bone and a shaft tapered to fit into a reamed medullary canal of the femur,20 prosthetic limbs consisting of a socket, shank, and foot for amputees,21 composite disc prostheses,22 and tailor-made, and computer-designed cranial implants used to minimize risks of brain surgery23 are examples of the many biomedical applications described in the literature. In these cases, the primary biological criterion for the materials employed was that they were "biocompatible" in the sense that there was minimal risk of inflammation of surrounding tissues.

The primary objective of creating biologically functional tissue-engineered scaffolding required the melding of the mechanical design and manufacturing strategies used in orthopedic and orthotic applications with the biological processes involved in tissue formation. In a series of journal articles, a CAD strategy was proposed that integrates advances in biology, biomedical engineering, information technology, and modern design and manufacturing processes in the design of scaffolds for tissue engineering application.24–27 The three components of the design strategy, labeled CATE, were characterized as "computer-aided tissue and bio-modeling, scaffold informatics and biomimetic design, and biomanufacturing for tissue and organ regeneration."

The first step in the CATE process is the use of 2D and 3D voxel-based medical imaging of the tissue or organ to characterize its structure. The voxel-based 3D images are then converted to a vector-based modeling environment needed for a CAD process. A library of CAD-based unit cells are created and used to construct a scaffold that mimics the shape, fibrous morphology, vascular channels, and pore size distributions within tissues and organs. Computer-based modeling is then employed to define an optimized CAD for a specific scaffold, which can then be incorporated into a CAD/CAM process for manufacture of the scaffold.
An in silico computational model for tissue-engineered scaffolds that uses finite element analysis (FEA) to incorporate solid and fluid mechanical principles with stochastic network modeling at hierarchical length-scales of tissue and cell and molecular dimensions was developed by Knothe Tate and colleagues.\textsuperscript{28–30} The human body was depicted as an ecosystem functioning at multiple hierarchical levels of length. The stresses and fluid flows within the poroelastic structure of the tissue at scale lengths of \([m^{-1}, m^{-2}]\) were characterized using classical solid and fluid mechanics. Stochastic network modeling was used within the pericellular networks and microporous matrices to characterize the movement of cells and molecules at the tissue-to-cell and cell-to-molecular levels \([m^{-6} \text{ to } m^{-7}]\). The mechanical and biological requirements were met by designing a porous scaffold with the required structural support and channels of interconnected pores through which cells, signals, and nutrients could be transported. Several versions of stereolithographic rapid prototyped scaffolds for bone replacement were manufactured to test the effectiveness of the design strategy in predicting the permeability of the manufactured scaffolds. Experimental measurements of the permeability of the scaffolds were comparable to values calculated using the in silico models, including several of the manufactured scaffolds that were predicted to be impermeable, thereby validating the design strategy as an economic way of determining appropriate designs for in vitro and in vivo studies. The stochastic modeling also ascertained the most effective morphology for promoting cell mobility and cell adhesion. The limitations of the CATE and similar approaches are associated with the difficulty of producing, from a CAD/CAM designed biodegradable scaffold, a true biomimetic reproduction of the complex fractal character of a naturally created ECM. The CAD/CAM-based unit cells used to build the mechanical structure of commercial products are limited by the Euclidean geometric shapes chosen and must be utilized in a manner that more adequately represents the topographical and fractal character of natural materials. Two of the more common CAM techniques utilized in manufacturing processes are rapid prototyping (RP) and electrospinning (ELSP). RP is a generic classification of processes used to fabricate a scaffold by building a sequence of thin layers, one at a time, to form a complete 3D structure directly from CAD/CAM sources. Cells and other biological substances that promote tissue in-growth can be incorporated into
the prefabricated layers during the assembly process. ELSP is an alternate route to mimicking the fractal character of natural tissues by spinning fibers from a biomaterial solution using a high voltage electrostatic field to form multiple layers of fibrous 3D nonwoven meshes, the orientations of which are controlled by controlling the path of the delivery nozzle or the direction of the electrical field. Meshes with fiber diameters ranging from micro- to nanometers can be produced, comparable to the dimensions of those in ECM of load-bearing tissues. Comprehensive presentations of the state of the art of these technologies are offered in *III: Fabrication, 11. Fabrication Methods of Tissue Engineering Scaffolds* (by V. M. Correlo, J. M. Oliveira, A. R. C. Duarte, A. Martins, A. L. Oliveira, J. Silva-Correia, R. L. Reis, and N. M. Neves); *12. Engineered Scaffolds: Materials and Microstructure from Nanostructures to Macrostructures for Tissue Engineering* (by V. G. Varanasi, P. S. Shiakolas, and P. B. Aswath); *13. Organ Printing and Cell Encapsulation* (by S. Tasoglu, U. A. Gurkan, S. Guven, and U. Demirci).

The creation of accurate 2D and 3D visual representations of tissue and organ structures for use in RP and other manufacturing techniques are generated by medical scanning devices such as magnetic resonance imaging (MRI) and computed tomography (CT). A combination of CT with positron emission tomography (PET), CT/PET, provides a more refined image that has the potential of measuring tissue functions such as blood flow. Electron beam CT (EBCT) scanners produce images in fractions of a second, even permitting the scanning of a beating heart, and diffraction-enhanced imaging (DEI) delivers more detailed images of soft tissue than is possible with other CT techniques. Imaging techniques are also employed in examination of diseased tissues and organs. Fluorescein angiography, for example, is used for imaging vascular patterns in the human retina for diagnosis of diabetic retinopathy and retinovascular occlusive disease. Image resolution of the vascular patterns of vessels of various dimensions present a fractal pattern that can be quantified and used to diagnose early-stage vascular disease in the human retina. Development of Fourier domain optical coherence tomography (FD-OCT) has been proposed as an improvement over fluorescein angiography due to improved sensitivity and acquisition time. Synchrotron radiation micro-CT has also been used for both morphological characterization and quantification of cell distributions down to the micrometer level of tissues, thereby
extending the range of imaging analysis from the whole tissue down into the cellular range. A comprehensive presentation of microvascular imaging methods for tissue engineering is given in *IV: Validation and Translation to the Application, 16. Microvascular Imaging Methods for Tissue Engineering* (by B. W. Thimm, S. Hofmann, and R. Müller).

*In vitro* and *in vivo* testing of the scaffold biomaterials is an indispensable step before acceptance by the medical profession and accrediting agencies of tissue-engineered scaffolding. Dr. Milena Fini and colleagues have pointed out the importance of *in vitro* and *in vivo* testing in determining the utility of biological therapies and biomaterials used for bone remodeling, humoral networks, and smart biomaterial technologies for osteoporosis mitigation.

A comprehensive presentation of the state of the art of *in vitro* and *in vivo* testing is offered in *IV: Validation and Translation to the Application, 14. In vitro and in vivo Testing* (by M. Fini, N. Nicoli Aldini, and P. Torricelli), while bioreactor strategies to develop neo-tissues *in vitro* are presented in *IV: Validation and Translation to the Application, 15. Bioreactor Strategies for in vitro Conditioning of Engineered Tissues* (by A. S. Goldstein and P. Thayer).

To mimic, more precisely, a true biomimetic reproduction of a naturally created ECM requires one to overcome the difference between an engineering approach and “nature’s way”:

While the engineer selects a material to fabricate a part to an exact design, nature goes the opposite way and grows both the material and the whole organism using the principles of self-assembly. This provides control of the material at all levels of hierarchy and is certainly a key to the successful use of polymers and composites as structural materials.

The transplant of a tissue-engineered bladder by Dr. Anthony Atala, mentioned at the beginning of this overview, was accomplished by utilizing the processes of *directed tissue assembly* that occur naturally in an ECM. Prior to the 1990s, the standard procedure for treating patients with neurogenic bladder disease was to remove the bladder and replace it with a section of the patient’s bowel, a dangerous surgery with serious limitations. Dr. Atala developed a technique of taking healthy cells from the patients’ diseased bladder, multiplying them *in vitro*, and then applying them to a balloon-shaped scaffold, made partly of collagen, with muscle cells on the outside and urothelial cells on the inside to line the urinary tract.
The bladder scaffold was then incubated until a functioning tissue was produced. The manufacture of a tissue-engineered scaffold for the bronchus connecting Claudio Castillo’s windpipe to her left lung was accomplished by using a decellularized donor windpipe that was recellularized with cells taken from Ms. Castillo. The creation of a beating heart by Ott, Taylor, and colleagues and the experimental transplantation of rat lungs by the team from Massachusetts General Hospital and Harvard University, also mentioned at the beginning of this overview, were accomplished by using similar decellularization/recellularization processes. Each of these approaches circumvented the problems of a top-down engineering design by inventing technologies that produce a 3D “scaffold” that more closely retains the original architecture of the nature-created ECM of the host tissue. Although the approach is a substantial advance in the field of tissue engineering, it will likely be limited by the availability of suitable donor tissues and complete donor organs. Thus, the search for natural and synthetic biomaterials that meet both the architectural and biological criteria for a functional tissue-engineered biomimetic scaffold has become a major objective of Tissue Engineering. A part of that objective has been the search for new molecular-modified biopolymers that elicit cellular responses at the molecular level by stimulating specific interactions with cell integrins that promote cell proliferation, differentiation, and the production of the host ECM. A thorough review of the materials used for scaffolds is offered in II: Materials for Scaffolds, 8. Biomaterials for Scaffolds: Synthetic Polymers (by L. Rojo, B. Vazquez, and J. San Roman); 9. Biomaterials for Scaffolds: Natural Polymers (by L. S. Wray and D. L. Kaplan); and 10. Hydrogels: Characteristics and Properties (by R. Barbucci and D. Pasqui).

Recent efforts to generate biocompatible biomimetic scaffolds have been to modify the surfaces in order to promote the appropriate biomechanical and chemical processes that support the tissue-specific cell functions that occur in the host material. Extensive research has focused on the structure and biological influence of the ECM on cell behavior. It has become clear that new technologies should focus on constructing well-defined microenvironments for cells at the nanoscale that match those of the host ECM. A tissue-engineered scaffold is most effective when all chemical and biomechanical processes progress by a bottom-up assembly of cell clusters matching that occurring in the ECM of the host tissue.
their compatibility depends upon the similarity of their morphology and composition, and on the cell adhesion, migration, growth, and differentiation processes encountered in each, the importance of understanding the central role natural ECMs play in the repair and regeneration of tissues and organs is critical. A natural, self-assembled ECM is formed by the secreted products of the cells that reside within tissues and consists of a mixture of structural proteins, hyaluronic acid, proteoglycans, growth factors, enzymes, and other components that are organized in a nature-determined morphology that is specific to a particular tissue. The role of the ECM and the biological processes that occur during the maintenance and repair of living tissues and organs are described in *I: Biological Design, 2. The Functional Role of Extracellular Matrix* (by E. Carletti, M. Stoppato, C. Migliaresi, and A. Motta).

Of primary importance in the choice of biomaterial for a scaffold is the response of the host tissue to the biomaterial implant. When it is implanted in the host tissue, it immediately adsorbs blood and other bodily fluids on its internal surfaces. Macrophages and dendritic cells of the host tissue interact with the protein-coated surfaces of the biomaterial and regulate the inflammatory or tolerogenic response to the scaffold by sensing its presence and providing the intracellular signals that determine acceptance or rejection of the foreign body. The present status of research on the biological processes of dendritic cells and macrophages and their potential for optimizing the host responses to the presence of biomaterial scaffolds is described in *I: Biological Design, 5. Host Response to Biomaterials* (by S. Srinivasan and J. E. Babensee).

In order to generate intracellular signals that indicate biocompatibility and an acceptably minimal inflammatory response to a biomaterial scaffold, the internal surfaces of a porous scaffold must be similar to that of the topography, composition, and molecular character of the ECM of the host tissue. Advances in an understanding of the mechanisms involved in the host response have led to techniques for modification of the surfaces of scaffold biomaterials using natural polymers such as heparin, hyaluronan, and hydrophilic polysaccharides to enhance their intrinsic bioactivity and specific interaction with cell surface receptors. In addition to molecular modification of the internal surfaces of a biomaterial, biocompatibility with the host also requires a similarity in the anisotropy and fractal character of the surfaces. These processes are
guided by the molecular composition and nanoscale topography of the scaffold surfaces. ELSP, for example, has been employed to mimic the circumferential and longitudinal mechanical properties of natural vessels to produce tubular, multilayer nanofibrous composites suitable as engineered vascular constructs. The rebuilding of tissues using a biomaterial scaffold requires the creation of an endothelium as a single layer of endothelial cells on the surfaces of the construct. The surface chemistry of the construct and the hemodynamic blood flow through the vascular channels determine the functionality of the resulting vasculature. There has been considerable research on optimizing the use of endothelialized biomaterials for creation of 3D vascularized constructs for tissue engineering applications and regenerative medicine, but many factors causing graft failure related to cell adhesion, matrix interactions that alter the phenotype of the endothelial cells and difficulty of predicting the responses to hemodynamic forces, and other factors have left many issues still to be dealt with. The deployment of biomaterial scaffolds for tissue vascularization is discussed in *I: Biological Design, 6. Scaffold Vascularization* (by L. E. Fitzpatrick, A. Lisovsky, E. C. Ciucurel, and M. V. Sefton).

Hydrogels are attractive for tissue-engineered scaffolding since they are biocompatible, hydrophilic and highly permeable, and their mechanical properties are easily manipulated. Thixotropic hydrogels are of special interest since they can be injected directly to a host site as a fluid, return to their original gel-like state *in situ*, and, when crosslinkable, become suitable as scaffolds for cell growth while allowing exchange of oxygen, nutrients, and soluble metabolites. The development of injectable hydrogel scaffolds containing natural polymers, soluble synthetic polymers and interpenetrating polymer networks (IPNs), and their adaptability for *in vivo* tissue formation have been widely employed. Aggregates of cells can be readily blended by direct assembly into the gel and then reorganized into larger complex patterns. Recognizing the importance of surface topography in promoting a biomimetic self-assembly of preformed cell clusters, a 3D tissue-engineered hydrogel-based scaffold was devised to direct the assembly process by partially restricting reorganization of the cell clusters on the free surface of a disposable form, acting as a template. The ability to also tailor the microenvironment within the gel leads to the possibility of incorporating small-scale chemical and physical gradients that act as signals to cells during

A primary characteristic of tissues and organs is their ability to adapt to changes in external stimuli. In choosing a biomimetic material for tissue engineering applications, researchers and practitioners are looking for biologically compatible materials that also can adapt positively to external stimuli by undergoing reversible physical or chemical changes at the host site. This has led to the search for *in situ* and “smart” materials that possess reversible phase transitions at body temperature and have a molecular structure with the necessary proportions of hydrophobicity and hydrophilicity that meet the requirements of a biomimetic response and can be introduced *in vivo* at the host site.

On behalf of the National Academy for Engineering, a panel entitled Grand Challenges in Biomaterials was convened at the 2010 Society for Biomaterials Annual Meeting in Seattle, Washington, USA, to consider challenges that, if successfully met, would significantly speed up the transition from commodity off-the-shelf biomaterials to bioengineered, biomaterial devices that both address patient needs and create new market opportunities. The six members of the panel offered six “challenges” that must be overcome to further advance these goals. The six challenges called for (1) “redefining biocompatibility to address new materials that tap into the body’s own reconstructive mechanisms and show improved healing and integration;” (2) finding an “appropriate rationale and use of biological experimental design and methodology in biocompatibility studies;” (3) a focus on *in vitro* applications and called for “smart new applications for smart polymers;” (4) a “grand challenge for moving medicine forward is full limb regeneration;” (5) a need to “harness the principles of developmental biology to control collective cell movement and differentiation *in vivo* and *in vitro;*” and (6) an all-encompassing challenge for “the necessity to continue to improve conventional “replacement” medicine by making better use of existing materials, developing better materials, producing better device designs, and improving surgical techniques” and, furthermore, suggested “the need to develop the materials and technologies that
optimize the inevitable transition from replacement medicine to regenerative medicine and tissue engineering.”

Much of the research, development, and transition to practice in the field of Scaffolds for Tissue Engineering during the 21st century have in fact been directed toward meeting the challenges exhorted by members of the above-mentioned panel. The efficacy of “top-down” designed functional tissue-engineered scaffolds will continue to advance by the increasing sophistication of engineering design principles, advances in manufacturing techniques, and the discovery of new “smart” biomaterials. The ability to build a biomimetic tissue-engineered scaffold will continue to advance using the principles of “bottom-up” self-assembly of the molecules and cells required by the host tissue or organ. Self-assembly at all dimensional scales has been substantially advanced by recent clarification of the intricate schedules of gene expression and proteome activity in cells and developing organisms. The same strategies used in the Human Genome project are now being applied to analyze the behavior of proteins and cells in tissues and organs. The spread of computer software designed for the analysis and importation of the data into in silico models of cell biochemistry has also enabled the study of numerous biological pathways for creating biomimetic scaffolding. One of the many techniques of building ordered assemblies of nanoparticles has been the directed self-assembly of nanoparticles carrying complementary strands of DNA molecules with flexible linkers of varying size. Tailoring the length of the linkages makes possible the self-assembly of nearly any material at a nanoscale level, and prescription of a specific thermal pathway makes possible the formation of a variety of ordered assemblies, thus opening up potential new avenues for studying biological interactions in a variety of 3D hybrid assemblies ranging from amorphous to highly ordered morphologies. The self-assembly of polymers, nanoparticles, and DNA can also be attained by controlling the evaporation of sessile droplets of a solute on a solid substrate to construct self-organized, hierarchically structured patterns at nanoscale levels.

The biomimetic restoration of damaged tissues requires the delivery of cells and biological factors, in vivo, to the scaffold surfaces to which they must adhere, organize, and develop in the same way as in the native tissue. Biosignals are incorporated to control the tissue-specific cell responses. One area of recent research has been the incorporation of natural ECM-derived peptides and pro-
teins to mimic natural cell-specific responses. Peptide and protein nanoscale patterning techniques are being studied to advance the technology of biomaterial scaffolding. The incorporation of short synthetic peptide sequences, either by surface modification or by bulk incorporation, to elicit a biomimetic response, and the effects of peptide immobilization on subsequent cell responses are also being pursued. The stabilization, in vivo, of relatively unstable fibroblast growth factors, (FGFs), by adsorbing or encapsulating them in a scaffold, is being investigated as a method of securing an acceptable level of biological activity in a targeted delivery system for biomimetic tissue regeneration. Tissue remodeling also requires cell-specific biological pathways that promote the appropriate metabolic pathways for the regeneration process. A morphogenic group of secreted lipid-modified glycoproteins (Wnts) that contribute to metabolic adaptation and regulate tissue maintenance and remodeling, for example, is being studied to map the linkage between Wnt signaling pathways and cellular metabolism. An implanted biocompatible scaffold often incurs a “tolerable,” but necessary irritation at the host site. Methods of controlling the extent of irritation by using naturally occurring peptides specifically at the target site have been shown to be effective and are critical in the acceptance of a tissue-engineered scaffold by medical regulatory agencies. A general introduction on aspects of molecular biorecognition will be discussed in I: Biological Design, 3. The Premise of Tissue Engineering: Molecular Recognition (by C. J. Pateman and J. W. Haycock). While the use of ECM macromolecules from natural sources in a tissue-engineered scaffold is often advantageous for biological recognition by a host tissue, the technique is limited by variations in materials isolated from mammalian sources. The use of artificial ECM proteins to modulate cellular behavior has been advanced as a means of avoiding such problems. Synthetic biomaterials are being designed that reproducibly mimic the functions of a natural extracellular microenvironment. Biological pathways to tissue regeneration will be discussed in I: Biological Design, 4. Principles and Biological Pathways To Tissue Regeneration: The Tissue Regenerative Niche (by R. Canciedda and C. Lo Sicco). The advance of potential medical products from the laboratory to the market place is presented in IV: Validation and Translation to the Application, 17. Tissue Engineering: Scope, Products, Commercialization Strategies (by A. J. Coury).
The three accomplishments mentioned at the beginning of this chapter are examples of the judicious use of fundamental research in developmental biology and bioengineering in the translation from the laboratory to medical techniques that will improve the quality of life and extend life expectancy of human beings. The inevitable advancement of these technologies will also create new social challenges for society and governments that will require a broadening of cooperation and participation beyond the bounds of science and engineering. This will be a critical challenge for humanity.

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Fuchs et al. 1 have reported that human microvascular endothelial cells (HDMEC) co-cultured with primary human osteoblasts (pOB) on three-dimensional porous hydroxyapatite, nickel-titanium, calcium phosphate, and silk fibroin scaffolds, considered as bone biomaterials, rapidly form microcapillary-like structures containing a lumen. The formation of vascular structures was seen just in the case where pOB were cocultured with the endothelial populations, was not enhanced using endothelial cells precultured alone. 2 The obtained pre-vascularized fibroin scaffolds, seeded with HDMEC and pOB, were then implanted in nude mice, showing the rapid anastomosis between the host vasculature and the in vitro generated vessel network, 3 a phenomenon that was especially evident in the cocultured condition. 2 During the in vitro cocultures, osteoblasts provided additional structural support for OEC-derived vessels, perhaps acting in a pericyte-like role, serving as stabilizing structural elements following implantation of pre-cultured vessels. 4 Results demonstrated the potential of pOB as a potent source of growth factors and extracellular matrix (ECM) that provide co-cultured endothelial cells with the necessary signaling for successful vascularization of tissue constructs in vivo. 2 These cells secrete bone-specific ECM and are known to produce a variety of factors that act on endothelial cells and promote their recruitment, survival, proliferation, and capillary formation. 5 Those factors include basic fibroblast growth factors, osteopontin, VEGF, and collagen type-I. Another potential mechanism whereby osteoblasts could promote scaffold vascularization
is through facilitating the permeation into the fibroin scaffold of host inflammatory cells, especially macrophages, which also appear to play a role in the vascularization process by their production of proangiogenic growth factors. Macrophages and multinucleated giant cells, which are involved in the degradation of silk fibroin micropatterns, produce angiogenic signaling molecules, such as VEGF, which could also contribute to scaffold vascularization.

The Role of Osteoblasts in in vitro Vascularization

Figure 1 Images of a representative HDMEC and HOS coculture on silk fibroin after 14 days in vivo. Image (A) is an HE-stained image of the implantation bed of the silk fibroin scaffold showing that the outer surface is surrounded by a large number of small lumen-containing vessels (arrows) and a few vessels with a larger lumen embedded within the fibers (arrowheads) that is identifiable by their content, that is, red blood cells, indicating perfusion. Image (B), staining with human-specific vWF shows the contribution of the HDMEC to the vascularization. Images (C and D) show the presence of vWF-stained human-specific vessels (arrow) and unstained murine vessels (arrowheads). Image (D) shows chimeric vessels containing human endothelial cells stained with vWF (arrow) integrated into vessels made up of primarily murine endothelial cells (arrowhead). The vessels in (C) and (D) are filled with erythrocytes. (A and B: 200¥ magnification and C and D: 600¥ magnification; scale bar, 100 mm). Reprinted from Ref. 3, Copyright (2010), with permission from Elsevier. The use of osteogenic cells could have implications in bone tissue engineering specifically, as the production of an osteogenic matrix would be concomitant with this induction of vascularization and could accelerate bone regeneration.


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Proteins constitute another source of hydrogels for tissue engineering scaffolds. Gels of proteins, such as collagen, fibroin, or fibrin have been widely proposed and also used to the tissue engineering of cartilage, intervertebral disks, skin, bone, and other tissues.
Proteins network

gels generally occur via hydrophobic interactions, hydrogen bonds,
or covalent bonds such as disulfide bonds. Gelation can be induced
by heat, pH lowering that promotes electrostatic interactions, shear
stresses, pressure, or ion addition.

1. Collagen

Collagen is the main component of the mammalian tissues, about 25%
of their weight, where it exploits mainly mechanical functions while
being a template for the ECM and tissue organization. It is mainly
produced by fibroblasts. Collagen is not only abundant in mammals,
but is also present in many organisms and making up almost the total
body of marine organisms, such as jelly fishes, or sea cucumbers, or
others. Native collagen has a fibrous structure that is dominated by
triple-helices conformation and stabilized by intramolecular bonds.

As such the molecule is not water-soluble. After denaturation and
hydrolysis, the quaternary and tertiary structure is lost and collagen,
now named gelatin, becomes water-soluble. Slightly crosslinked
collagens (e.g., immature collagen that is present in some organisms)
can also be solubilized in acidic environments.

Gelatin has an incredibly large number of uses in different fields.

In biomedicine, it is used as coating of cell culture plates, and also in diagnostics, in immunohistochemistry.

Most of the gelatin originates from mammals, mainly pig skin. In recent years, gelatin from fishes has attracted increasing attention and jelly fishes, sea cucumber, or fish scale-derived gelatins have been investigated, also for tissue engineering applications. 1,2

As well discussed, 3 among the 29 different types of collagen that are present in mammals, collagen type I is the most suitable Protein-Based Hydrogels for implantation since only few persons possess specific immunity against it, that can be easily revealed by serologic allergenic tests.

Collagen is degraded by collagenase, an enzyme present in our body, which breaks the peptide bonds in collagen.

Collagen gels can be easily prepared by heating collagen type I solutions. Heating generates assembling of the collagen monomers into fibrils that further aggregate in bundles or fibers, with fibrils morphology and aggregation controlled by pH, ionic
strength, and temperature. 4

Composites of poly(lactic acid-co-e-caprolactone) (PLAC) and collagen gels have been used as scaffolds for the regeneration of the bladder wall. 5 Collagen gels contract when seeded with fibroblasts and other connective tissue cells (Fig. 1). 6 This ability of the cells is the basis of the modeling and remodeling of tissues. The ability of tissues to remodel should be always considered in the tissue regeneration pathway.

Figure 1 Sequence showing how a fibroblast cell extends and contracts over a collagen fiber, orienting itself along the long axis of the fiber and extends lamellipodia along the fiber scale bar, 5 mm. Reprinted by permission from Macmillan Publishers Ltd: Nature Cell Biology (Ref. 6), copyright (2005).

2. Fibroin

As reported in the chapter on natural polymers, fibroin from silkworm silk acquires progressive interest for applications in tissue engineering. Fibroin gels can be easily prepared with different methods, such as sonication, acidification, shear stresses application, or supercritical CO 2 . The physical characteristics of fibroin gel depend on the concentration of the starting water solution and on the method used for gelation. For instance, acidification
of water

solutions form flowable gels, while more compact and stiffer gels can

be obtained by supercritical CO$_2$ treatments or sonicating higher

fibroin concentration water solutions. Gelation occurs via b-sheets

and intermolecular bonds formation. Injectable fibroin gels have

been used to induce bone repair in critical size defects in rabbits or
to encapsulate cells.

For instance, Fini et al. (Fig. 2), have shown that fibroin gels

can induce critical size bone defect regeneration after four weeks,

with the formation of smaller bone trabeculae, well integrated with

the existing bone. A full remodeling process occurring within three

months reconstituted the normal anatomy of the newly generated

bone.

Figure 2 A critical size defect in the femur condyle of a rabbit filled with a fibroin gel. (a) The hole populated by small bone trabeculae after four weeks; OB, original bone; NB, new bone. (b) Mineralizing new bone with osteoblasts lining the trabecular surface (arrows) near the center of the defect. (c) Remodeling after 12 weeks and the recovery of the morphology of the original bone. Reprinted from Ref. 9, Copyright (2005), with permission from Elsevier.

3. Fibrin
The proteolitic cleavage by thrombin of fibrinopeptides transforms fibrinogen in fibrin, a fibrous protein involved in the coagulation of the blood. The resulting network of fibers forms hydrogels even at very low volume fraction of polymer. The characteristics (rheology and stability) of the gels can be modulated by changing fibrinogen and thrombin concentration.

Unlike synthetic hydrogels, fibrin can directly interact with cells being provided with two pairs of RGD sites. Fibrin gels have been long used for many applications. Examples are drug release, and in the field of tissue engineering, the regeneration of adipose tissue, bone, cartilage, liver, nervous tissue, skin, tendons, ligaments, and cardiovascular tissue engineering.

Figure 3 (A) Fibrin gel after polymerization. (B-D) SEM images of the nanofiber network structure of the fibrin, which enables the gentle embedding of cells, with a vascular smooth muscle cell (SMC) shown in (D) within the fibrin network (Image courtesy: InTech).

Figure 4 A vascular graft made of (A) macroporous mesh of poly(D,L) lactic acid, (B) embedded into a fibrin pre-seeded with SMC, and (C) implanted in a ovine carotid model (Image courtesy: InTech).

4. Other Systems Uriel et al. prepared gel matrices starting from solutions of proteins and glycoproteins from soft tissues, showing that the gel matrices
supported tissue-specific, integrin-mediated cell growth, and EC network formation. Ritzoulis et al. investigated milk protein-derived gels for bone tissue engineering. Santin et al. also investigated the use of soybean gels and demonstrated that soy flour is an inexpensive source for the preparation of gels with different physicochemical properties, nontoxic behavior, and intrinsic bioactivity on cells.

5. Remarks

Tissue engineering scaffolds are usually designed and fabricated to match as closely as possible the native tissue properties and architecture. A general opinion is that the more a scaffold resembles the ECM, the more successful the scaffold will be. It is generally true; however, the fact that a scaffold should always accurately match properties or architecture of the target tissue can be controversial. An example, reported in this book, is the ability of some gels to promote complete and physiologically consistent bone regeneration after their insertion or injection in the defect. Spontaneous regeneration of natural tissues occurs via the remodeling of the ECM.
matrix. The process of regeneration always involves remodeling,
and in favorable conditions the newly formed tissue remodels and
assumes the target tissue morphology, structure, composition, and
properties. Moreover, the implanted scaffold undergoes modification
after implantation and its structure, composition, and properties
change.
The regeneration of a tissue is a developmental process of constructive tissue remodeling, and scaffolds should promote it and
be compatible with it.
Importantly, our concept of a scaffold includes both the 3-dimen
sional traditional geometrically defined construct and the newer
injectable material, which does not provide a distinct macroscopic
architecture but still provides a controlled microenvironment for the
cells. 18


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Cells produce the extra cellular matrix (ECM), which in turn makes up cartilage, bone, ligament, and all other tissues. At the same time, the ECM constitutes the scaffold for cells, provides structural support to them, provides binding sites for cells, and is a reservoir of nutrients or biological molecules that are used in the continuous remodeling process of the tissues or in their fabrication.
Making scaffolds means mimicking the role and the functions of ECM. However, even if man-made scaffolds can get close to the morphology and mechanical properties of ECM, mimicking its functional properties is not easy. On the role of ECM as a scaffold, readers are invited to consult, among others, the paper of Badylak. Supposedly, decellularized ECM (Fig. 1) could be the ideal scaffold for tissue regeneration, provided that the signaling sites are preserved, that is, they provide sites for the biomolecular recognition. The decellularization process should remove only cells, lipids, and all protein except structural proteins (e.g., collagen or elastin), without affecting their integrity. However, during decellularization, besides the cells being removed, generally the biochemical structure, composition, physical properties remodeling response, and functional outcome of the resulting scaffold also gets affected by the mechanical, chemical, and enzymatic processes that are used for the decellularization. In addition, ineffective or incomplete decellularization can influence host response to implantation, macrophage response, and remodeling outcome. Decellularized Tissues as Scaffolds Cells from biopsy Bioreactor Decellularization heart Decellularization process Validation Implantation Discarded

Figure 1 Schematic diagram of an organ decellularization-application pathway. Adapted from Ref. 4.
In addition to the methods reported in Fig. 2, supercritical CO$_2$ extraction has been developed that does not require the use of surfactants. The application of supercritical CO$_2$ provides a clean technology to obtain acellular ECM with a good guarantee of absence of the pathogens and proteins, avoiding the use of solvents that could be retained in the ECM after the drying process.

However, the application of supercritical technologies has some limitations, the most important being the relatively limited dimensions of the chamber or reactor for the application of supercritical conditions. The application of this process is very attractive if water is absent. Otherwise, the presence of small quantities of water produces the formation of carbonic acid from CO$_2$ and therefore, the elimination of water by washing with organic solvents previous to the treatment with CO$_2$ is necessary. Nevertheless, decellularized tissues and organs forms have been successfully used for clinical needs in humans, such as for trachea regeneration by using a decellularized human trachea, tested in animals for blood vessels, and even approved for commercialization.
(e.g., for burn wound covering or heart valves) (Table 1).

For the sake of clarity, pig skin for burn treatments and porcine heart valves are an example of wide and successful use of decellularized tissue, however, they are not scaffolds for engineering new tissue.

Recently, a decellularized pig kidney has been proven to be effective for the regeneration of the kidney in pigs. 10

Figure 2 Example decellularization protocols for (A) thin laminates such as pericardium, (B) thicker laminates such as dermis, (C) fatty, amorphous tissues such as adipose, (D) composite tissues or whole simple organs such as trachea, and (E) whole vital organs such as liver. Arrow lengths are proportional to exposure times. (F) Appearance of rat liver subjected to decellularization: (left to right) before, during, and after decellularization; decellularized liver perfused with blue dye. (G) Photomicrographs after nuclear staining reveal the removal of cells: (left to right) native rat liver, HE stain; decellularized liver-ECM, HE stain; native rat liver DAPI stain; liver-ECM, DAPI stain. Scale bars: 50 mm. Reprinted from Ref. 5, Copyright (2011), with permission from Elsevier.

Table 1 Some commercial decellularized materials

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<th>Product</th>
<th>Company</th>
<th>Material Type</th>
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<td>Alloderm ®</td>
<td>LifeCell™</td>
<td>Human skin</td>
<td>Natural</td>
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<tr>
<td>Axis™ dermis</td>
<td>Mentor</td>
<td>Human dermis</td>
<td>Natural</td>
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<tr>
<td>Bard Dermal</td>
<td>C R Bard</td>
<td>Cadaveric human dermis</td>
<td>Natural</td>
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<tr>
<td>CuffPatch™</td>
<td>Biomet Sports Medicine</td>
<td>Porcine small intestinal submucosa (SIS)</td>
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<tr>
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<td>Pegasus Biologicals</td>
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<td>Dura-Guard ®</td>
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