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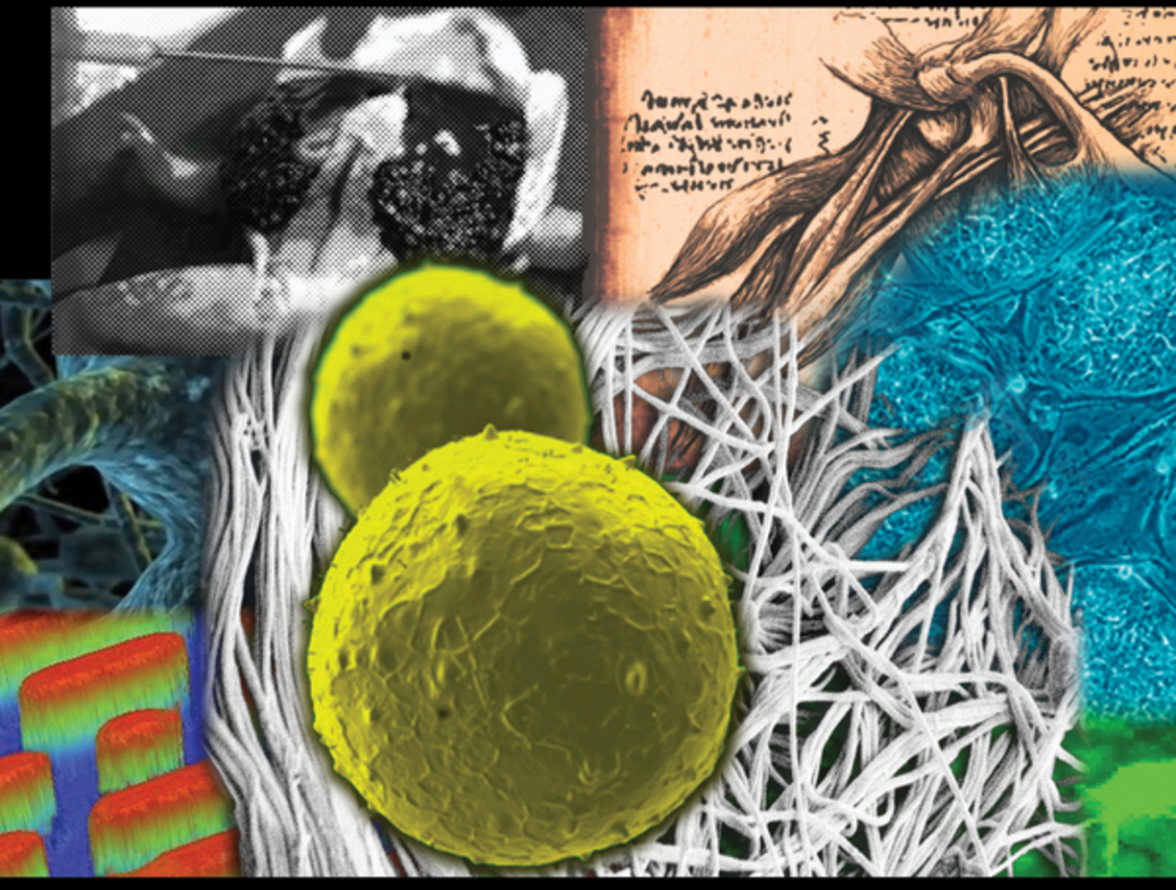
Bio-inspired Materials for Biomedical Engineering

Edited By **Anthony B. Brennan & Chelsea M. Kirschner**



With a foreword by
Sang Jin Lee and Anthony Atala, Wake Forest Institute for Regenerative Medicine

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BIO-INSPIRED MATERIALS FOR BIOMEDICAL ENGINEERING

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Edited by

ANTHONY B. BRENNAN

CHELSEA M. KIRSCHNER

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CONTENTS

CONTRIBUTORS

Marta Alves da Silva, 3B's Research Group – Biomaterials, Biodegradables, and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal; ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

Michael Ansorge, Universität Leipzig, Institute of Biochemistry, Leipzig, Germany

Ezgi Antmen, BIOMATEN, METU Center of Excellence in Biomaterials and Tissue Engineering, Department of Biotechnology, Ankara, Turkey

Anthony Atala, Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, USA

Stephen F. Badylak, McGowan Institute for Regenerative Medicine, Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA

Erkan Türker Baran, BIOMATEN, METU Center of Excellence in Biomaterials and Tissue Engineering, Ankara, Turkey

Liming Bian, Division of Biomedical Engineering, Department of Mechanical and Automation Engineering, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

Anthony B. Brennan, Department of Materials Science and Engineering, J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL, USA; Sharklet Technologies, Inc., Aurora, CO, USA

Ana Costa-Pinto, 3B's Research Group – Biomaterials, Biodegradables, and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal; ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

Christopher L. Dearth, McGowan Institute for Regenerative Medicine, Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA

Tuğba Dursun, BIOMATEN, METU Center of Excellence in Biomaterials and Tissue Engineering, Department of Biotechnology, Ankara, Turkey

Adam J. Engler, Material Science Program, Department of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California–San Diego, La Jolla, CA, USA

Menekse Ermis, BIOMATEN, METU Center of Excellence in Biomaterials and Tissue Engineering, Department of Biomedical Engineering, Ankara, Turkey

Adam W. Feinberg, Department of Biomedical Engineering, Department of Materials Science and Engineering, Carnegie Mellon University, Pittsburgh, PA, USA

Anthony W. Frei, Diabetes Research Institute, Leonard M. Miller School of Medicine, University of Miami, Miami, FL, USA; Department of Biomedical Engineering, College of Engineering, University of Miami, Coral Gables, FL, USA

A. Jon Goldberg, Reconstructive Sciences, University of Connecticut Health Center, Farmington, CT, USA

Vasif Hasirci, BIOMATEN, METU Center of Excellence in Biomaterials and Tissue Engineering, Departments of Biological Sciences, Biomedical Engineering, and Biotechnology, Ankara, Turkey

Jay D. Humphrey, Department of Biomedical Engineering, Yale University, New Haven, CT, USA

Emily Jacobs, Reconstructive Sciences, University of Connecticut Health Center, Farmington, CT, USA

Quentin Jallerat, Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, USA

Benjamin G. Keselowsky, J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL, USA

Hye Young Kim, Departments of Chemical and Biological Engineering and Molecular Biology, Princeton University, Princeton, NJ, USA

Chelsea M. Kirschner, Sharklet Technologies, Inc., Aurora, CO, USA

Elizabeth W. Kollar, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Liisa T. Kuhn, Reconstructive Sciences, University of Connecticut Health Center, Farmington, CT, USA

Elaine L. Lee, Department of Biomedical Engineering, Boston University, Boston, MA, USA

Sang Jin Lee, Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, USA

Christopher A. Lemmon, Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA, USA

Jamal S. Lewis, J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL, USA

Albino Martins, 3B's Research Group – Biomaterials, Biodegradables, and Biomimetics, University of Minho, Headquarters of the European Institute of

Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal; ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

Eike Müller, Leibniz Institute of Polymer Research Dresden, Max Bergmann Center of Biomaterials, Dresden, Germany

Celeste M. Nelson, Departments of Chemical and Biological Engineering and Molecular Biology, Princeton University, Princeton, NJ, USA

Nuno M. Neves, 3B's Research Group – Biomaterials, Biodegradables, and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal; ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

Matthew G. Ondeck, Material Science Program, University of California–San Diego, La Jolla, CA, USA

Dennis P. Orgill, Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Shawn C. Owen, The Donnelly Centre for Cellular and Biomolecular Research, Department of Chemical Engineering and Applied Chemistry, Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada

Tilo Pompe, Leibniz Institute of Polymer Research Dresden, Max Bergmann Center of Biomaterials, Dresden; Universität Leipzig, Institute of Biochemistry, Leipzig, Germany

Rui L. Reis, 3B's Research Group – Biomaterials, Biodegradables, and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal; ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

James F. Schumacher, J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL, USA

Molly S. Shoichet, The Donnelly Centre for Cellular and Biomolecular Research, Department of Chemical Engineering and Applied Chemistry, Institute of Biomaterials and Biomedical Engineering, Department of Chemistry, University of Toronto, Toronto, Ontario, Canada

David D. Simon, Department of Biomedical Engineering, Yale University, New Haven, CT, USA

Cherie L. Stabler, Diabetes Research Institute, Department of Surgery, Leonard M. Miller School of Medicine, University of Miami, Miami, FL, USA; Department of Biomedical Engineering, College of Engineering, University of Miami, Coral Gables, FL, USA

John M. Szymanski, Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, USA

Roger Y. Tam, The Donnelly Centre for Cellular and Biomolecular Research, Department of Chemical Engineering and Applied Chemistry, Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada

Hongjun Wang, Department of Chemistry, Chemical Biology and Biomedical Engineering, Stevens Institute of Technology, Hoboken, NJ, USA

Carsten Werner, Leibniz Institute of Polymer Research Dresden, Max Bergmann Center of Biomaterials, Technische Universität Dresden, Center for Regenerative Therapies Dresden, Dresden, Germany

Joyce Y. Wong, Departments of Biomedical Engineering and Materials Science and Engineering, Boston University, Boston, MA, USA

Meng Xu, Department of Chemistry, Chemical Biology and Biomedical Engineering, Stevens Institute of Technology, Hoboken, NJ, USA

PREFACE

Natural materials exhibit highly sophisticated properties selected through evolution to achieve specific functions efficiently. As engineers, biological scientists, and physicians strive to recapitulate natural biological processes, such as wound healing or tissue regeneration, they incorporate bio-inspired approaches. These strategies have been implemented in the rational design of biomedical devices and biomaterials both to treat patients in the clinic and to probe the fundamental mechanisms of cellular interactions with biomaterials. In this effort, our community endeavors not only to copy the complex hierarchical structures present in nature, but to harness the power of natural processes to create dynamic, bioactive materials.

This book, the first in the Wiley–Society For Biomaterials book series, aims to introduce the reader to bio-inspired strategies that provide elegant solutions for contemporary biomedical engineering challenges. The intended audience is multidisciplinary and includes students and practitioners of materials science, engineering, biology, chemistry, physics, medicine, dentistry, and veterinary medicine. This level of diversity is pervasive throughout the biomaterials community and essential for innovation in that it allows researchers to approach modern biomedical engineering challenges from a variety of perspectives. The chapters that comprise this book originate from authors all over the world with expertise from a variety of disciplines and specialties. The text is divided into two parts—Engineering Bio-inspired Material Microenvironments and Bio-inspired Tissue Engineering—in an effort to introduce the reader to fundamental concepts in biomaterials science and engineering, as well as provide a perspective on the clinical application of these technologies.

These goals could only be achieved with a tremendous amount of support and involvement from the biomaterials community. First, we must introduce and acknowledge the Society For Biomaterials as the sponsor and inspiration for this book. The theme for this text originated as the topic for a general session organized by the editors at the annual Society For Biomaterials meeting in Orlando, Florida. The Society For Biomaterials provides the opportunity for engineers and scientists from industry and academia to collaborate with physicians and business professionals to promote advancement in all aspects of biomaterial science, education, and professional standards to enhance human health and quality of life. The editors also acknowledge and extend their sincere gratitude to the authors who generously devoted their valuable time to contribute their expert knowledge and experience to

this book. The active and professional support of the editorial and production staff at Wiley is also appreciated.

We hope that this book will empower the reader to think beyond current paradigms when relating bio-inspired engineering concepts to the translation of biomaterials science into medicine, and in turn, contribute to the evolution and expansion of bio-inspired materials in medicine.

Anthony B. Brennan
Chelsea M. Kirschner

INTRODUCTION

SANG JIN LEE and ANTHONY ATALA

Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, USA

Restoration and maintenance of normal function of injured or damaged tissues and organs with biological substitutes is the primary objective of tissue engineering, a major component of regenerative medicine that adheres to the standards established in the areas of cell transplantation, materials science, and engineering. Engineered tissue constructs that are composed of biomaterial scaffolds preseeded with tissue-specific cells are among the most promising approaches to generate biologically and/or mechanically functional tissue replacements. Clinical applications of tissue engineering and regenerative medicine technologies, however, have been relatively restricted due to the limitation in clinically approved biomaterials. The varieties of biomaterials, which have been developed in recent years, have encountered delays in translation to clinical practice. Many investigators have resorted to using biodegradable synthetic polymers that were first approved for use in humans more than 30 years ago. During normal development, tissue morphogenesis is greatly influenced by the interactions between cells and the extracellular matrix (ECM) proteins; however, these simple polymers, which have been used historically to provide architectural support for neotissue development, poorly mimic the complex interactions between tissue-specific cells and the tissue-specific ECMs that promote functional tissue regeneration. Consequently, tissue engineering and regenerative medicine strategies will advance as biomaterials that actively participate in functional tissue regeneration are developed.

Nature provides numerous systems that possess exceptional properties and performance that might be replicated for many biomedical applications. Thus, scientists have observed phenomena of nature, learned the principles, and incorporated various characteristics to mimic biological systems into materials. For example, the fabric hook and loop fastener was inspired by the seeds of the burdock plant. Adhesive biomaterials have been fabricated by using microfabrication techniques inspired by the feet of the gecko, which has extraordinary climbing ability. Multifunctional biomaterials that mimic the chemical composition, physical structure, and biologically functional moieties of natural living systems could contribute to the development of new biomaterials for tissue engineering applications. Accordingly, recent progress in tissue engineering strategies has led to a paradigm shift in biomaterials

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research, whereby the concepts of bio-inspiration and biomimetics play a more active role in small-scale structures and their time-dependent biological interactions with the host.

Throughout this book, the bio-inspired materials are defined as types of biomaterials that contribute to tissue engineering and regenerative medicine strategies to achieve multifunctional and integrated tissues or organs, which incorporate biological, chemical, mechanical, topographical, and electrical cues derived from nature, as well as offer space as a scaffold. By reproducing principles or structures of biological systems, bio-inspired materials offer several aspects of higher level integration in biological systems: sophistication, miniaturization, hierarchical organizations, hybridization, resistance, and adaptability, which could actively participate in functional tissue regeneration in tissue engineering applications. This collection of contributions is divided into two overarching themes: Part 1, Engineering Bio-inspired Material Microenvironments and Part 2, Bio-inspired Tissue Engineering.

In Part 1, three major approaches to bio-inspiration are introduced: (1) ECM-mimetic bioactive materials, (2) physicochemical signals for controlling cell fates, and (3) scaffolds derived from natural materials. In the tissue regeneration process, the interactions between cells and tissue-specific ECMs are critical since cell attachment to the ECMs regulates various cellular functions such as proliferation, migration, and differentiation. A variety of synthetic biodegradable polymers have been used as tissue-engineered scaffolds because they possess adjustable mechanical properties, biodegradability, and functionality. Synthetic polymers, however, often lack biological recognition. Naturally derived hydrogels, such as fibrin and collagen, have biological functions that enhance cell adhesion, proliferation, and differentiation, which are lacking in synthetic polymers. Therefore, synthetic polymers have been modified to mimic ECM functions using short peptide sequences derived from the bioactive domains of ECM components, including adhesive peptides, enzyme-sensitive peptides, and growth factors. Bioactive molecules (or signaling molecules), including proteins and small molecules, involved in tissue regeneration also play an important role in controlling the microenvironment *in vivo*. Chemotactic signals from bioactive molecules are responsible for inducing host cell mobilization; moreover, an anatomic destination is identified according to certain concentration gradients of chemicals produced at injured sites within the microenvironment. Bioactive molecules are able to regulate host cell migration, proliferation, and differentiation, and allow cells to interact via specific receptors for chemical recognition with their surrounding microenvironment. Thus, incorporation of a suitable bioactive molecule through the design of a tissue-engineered scaffold can promote tissue regeneration by stimulating the transplanted cells or adjacent host cells. The first chapter describes how ECM-inspired chemical cues can be incorporated into scaffolds for tissue engineering while the second details how dynamic materials can be used to recapitulate signals from the ECM.

Cells are exposed to tissue-specific microenvironments *in vivo*, and they respond to numerous chemical and physical stimuli. As a result, cellular functions like cell adhesion and proliferation, protein synthesis, and cytoskeletal architecture are critically influenced by the microenvironment of the cells. To maintain the phenotype expression and differentiation of tissue-specific cells, numerous cues have

been applied to biomaterial scaffolds to mimic natural ECM microenvironments. Especially, the growth and specific differentiation of stem cells (embryonic or adult) are significantly affected by their microenvironments, including chemical, topographical, mechanical, and electrical cues. The interactions of cells with biomaterials are critically important for the successful outcome of tissue engineering applications. Thus, the behavior of cells grown on a biomaterial surface, including adhesion to the material, development of appropriate cellular structures, and maintenance of proper cell phenotype and function, must be investigated in order to obtain insight into the characteristics of the biomaterial. Substrate modulus and external mechanical stimuli are important for maintaining phenotype, controlling stem cell differentiation, and establishing functional tissues. Tissues can experience compressive forces or tensile forces, such as mechanical loading, or stretch and fluid-applied forces, such as shear flow. These phenomena are explored in two chapters in Part 1.

Cells also encounter topographical cues in the form of the physical features of their surrounding microenvironment. The topography of the surface of a biomaterial can directly influence cell adhesion and proliferation, which further affects cellular functions. For this reason, one chapter in the first section focuses on engineering bio-inspired topographic cues into materials for biomedical engineering and the resulting cellular responses. Finally, this section is completed with two distinct chapters that describe how naturally derived materials can be exploited as tissue engineering scaffolds, and one chapter is devoted to the role of one specific ECM protein, fibronectin.

In Part 2 of Bio-inspired Materials for Biomedical Engineering, these approaches to engineering bio-inspired cellular microenvironments are applied to tissue engineering. The chapters in this section highlight the implementation of bio-inspired design to create epithelial tissue, cardiac tissue, musculoskeletal tissue, and connective tissue and to elicit specific immune responses. Bio-inspired materials are desirable as tissue-engineered scaffolds because they mimic the native microenvironment of the ECM. Development of tissue engineering strategies continues to be a critical component of the research pursuits in the field of regenerative medicine. Future advances in tissue engineering strategies rely on the development of bio-inspired materials that actively participate in functional tissue regeneration. Bio-inspired materials could provide biological, chemical, mechanical, and structural functions that are inspired from nature. Desirable bio-inspired materials could be designed as scaffolds that mimic the natural biological system and integrate the necessary structural and biological properties. Solid understanding of materials science combined with extensive knowledge of the clinical challenges and cell biology is vital for the development of clinically applicable biomaterials to be used in tissue engineering. Therefore, interdisciplinary collaboration between material scientists, engineers, cell biologists, physiologists, and clinicians should be encouraged to develop novel bio-inspired materials for tissue-engineering applications that might enhance or improve current regenerative medicine therapies.

Acknowledgment

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PART I

ENGINEERING BIO-INSPIRED MATERIAL MICROENVIRONMENTS

CHAPTER 1

ECM-INSPIRED CHEMICAL CUES: BIOMIMETIC MOLECULES AND TECHNIQUES OF IMMOBILIZATION

ROGER Y. TAM and SHAWN C. OWEN

The Donnelly Centre for Cellular and Biomolecular Research, Department of Chemical Engineering and Applied Chemistry, Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada

MOLLY S. SHOICHET

The Donnelly Centre for Cellular and Biomolecular Research, Department of Chemical Engineering and Applied Chemistry, Institute of Biomaterials and Biomedical Engineering, Department of Chemistry, University of Toronto, Toronto, Ontario, Canada

1.1 INTRODUCTION

The extracellular matrix (ECM) is a complex environment that provides chemical and physical support to cells, Figure 1.1 [1,2]. The composition of native ECM differs based on its location within the body [3–6], but it is generally comprised of proteins (fibronectin, laminin, and collagen), polysaccharides (hyaluronan and chondroitin sulfate proteoglycans [CSPGs]) and various growth factors [6]. Components of the ECM play important roles in controlling cell function. Molecules such as collagen [7] and elastin [8] function as the structural scaffold to support cell growth, whereas fibronectin, laminin, glycosaminoglycans (GAGs), and growth factors act as ligands to promote cell adhesion, proliferation, differentiation, and migration [9].

Cells can remodel the ECM in a dynamic fashion [9,10]. For example, cells can secrete proteases that can degrade the ECM to promote cell migration, which is important in tissue repair, such as neuroblast migration following traumatic brain injury (TBI) [11], as well as in disease states, such as cancer metastasis [12]. Cells

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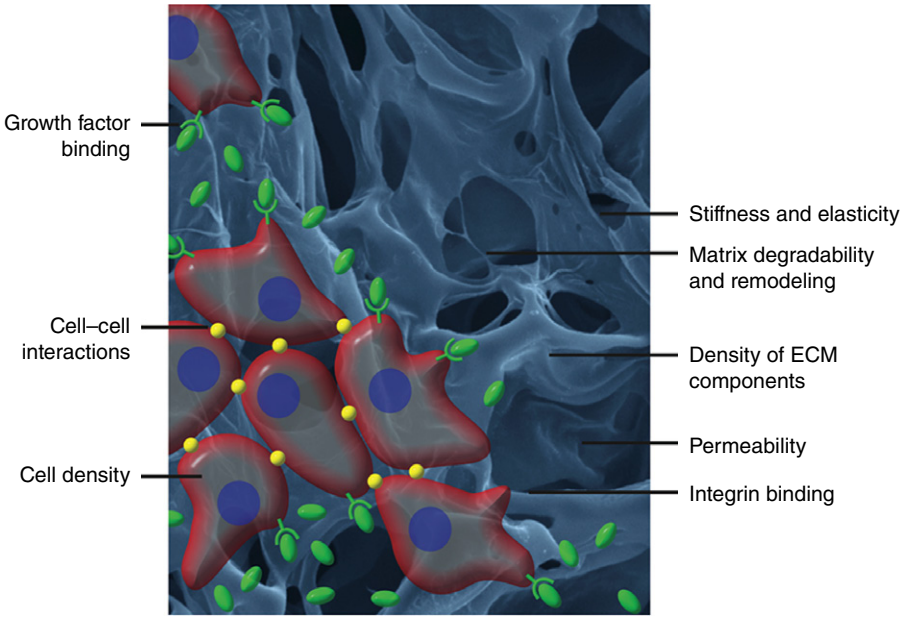

FIGURE 1.1 The complex 3-D cellular environment provides mechanical and biochemical signals that guide cell function. The components of the ECM dictate the stiffness of matrix and the types of cell–matrix interactions. The matrix composition determines the ease with which nutrients diffuse through tissues and the ability with which cells migrate through the matrix. Nonstructural factors such as cell density, cell–cell interactions, and bound or secreted signaling proteins are important in guiding cell differentiation and function. (Reproduced with permission from Owen, S.C., Shoichet, M.S. *Journal of Biomedical Materials Research A* **2011**, *94A*(4). Copyright 2013 Wiley Periodicals Inc.)

can also secrete their own ECM molecules on top of the existing ECM to provide new cues affecting both self and neighboring cells [10].

The increased understanding of the role of native ECM on cellular function and interactions has resulted in extensive research into biomimetic materials for applications in tissue engineering [13,14]. Hydrogels represent a class of biomaterials that have been used for this purpose. These highly hydrated polymers provide structural scaffolds and permit diffusion of molecules throughout. Matrigel® (BD Biosciences, San Jose, CA), a decellularized ECM derived from the Engelbreth–Holm–Swarm (EHS) mouse sarcoma, is a common hydrogel used to mimic the three-dimensional (3-D) properties of the ECM [15]. This material has been shown to promote various bioactivities such as cell adhesion, differentiation, viability, and invasion in a variety of cell types; however, for studies that require a more defined 3-D environment (such as those for mechanistic elucidation studies), the use of Matrigel® is nonideal as it is ill-defined in composition and the results are often difficult to reproduce. As such, a bottom-up approach is desirable where researchers begin with a blank palette in terms of cellular interactions and then *paint* in desirable

features, such as cell adhesion, proliferation, and migration through both chemical and physical designs. Efforts to synthesize biomimetic ECMs with defined components were significantly advanced by the discovery that short peptide sequences (e.g., RGD, YIGSR, IKVAV, etc.), derived from native ECM proteins (fibronectin and laminin, respectively), promote cell adhesion and outgrowth. It was shown that RGD interacted with extracellular integrin receptors with affinity similar to that of native fibronectin [16]. Since this discovery, a large number of studies have been conducted to immobilize this and other biomimetic sequences to various biomaterials with the intention of promoting cell adhesion on nonadherent surfaces and biomaterials, thereby increasing the posttransplantation cell viability in tissue regeneration applications, and studying cell behavior in model biomimetic systems.

This chapter is focused on recent advances in the techniques used to incorporate ECM-inspired chemical signaling molecules into different hydrogels, and their effects on cellular interactions in 3-D. While similar approaches are used in multiple areas of biology, we highlight many examples applicable to neurobiology.

1.2 DEVELOPMENT AND IMMOBILIZATION OF BIOMIMETIC CUES IN 3-D BIOMATERIALS

The discovery that short peptide sequences showed comparable activity to their respective native ECM proteins from which they were derived has resulted in significant efforts to design peptide-modified biomaterials with which to study cellular interactions [17,18]. Biomaterial modification with these peptide sequences (typically 3–10 amino acids) results in inherently better-defined systems than the corresponding protein-modified systems due to the shorter sequence length and resulting 3-D structure. To take full advantage of ligand-containing biomaterials to study complex cellular interactions, it is imperative that the ligand is chemically conjugated to the biomaterial in a reproducible and specific manner in order to optimize cellular interaction. For example, conjugation at the active site of the peptide may diminish receptor binding and therefore limit bioactivity. An important consideration in designing biomimetic molecules is to include a specific functional group that has a selective chemical reactivity toward the material to which it will be conjugated.

The emergence of *click* chemistry as a method to conjugate molecules to biomaterials has proved to be a powerful technique to allow efficient conjugation with both defined chemical reactivities and orientation [19–22]. These orthogonal reactions are specific and occur with high yield and efficiency. While detailed discussion about this topic is beyond the scope of this chapter, Figure 1.2 shows a brief summary of different conjugation reactions that have been used to immobilize various peptides and proteins to biomaterials. The following section will describe the conjugation of various peptides to different biomaterials using these techniques. While most chemical conjugations have focused on the irreversible conjugation of molecules, recent work has enabled a versatile approach to forming reversible conjugations, which has the potential to synthesize dynamic biomimetic systems [23].

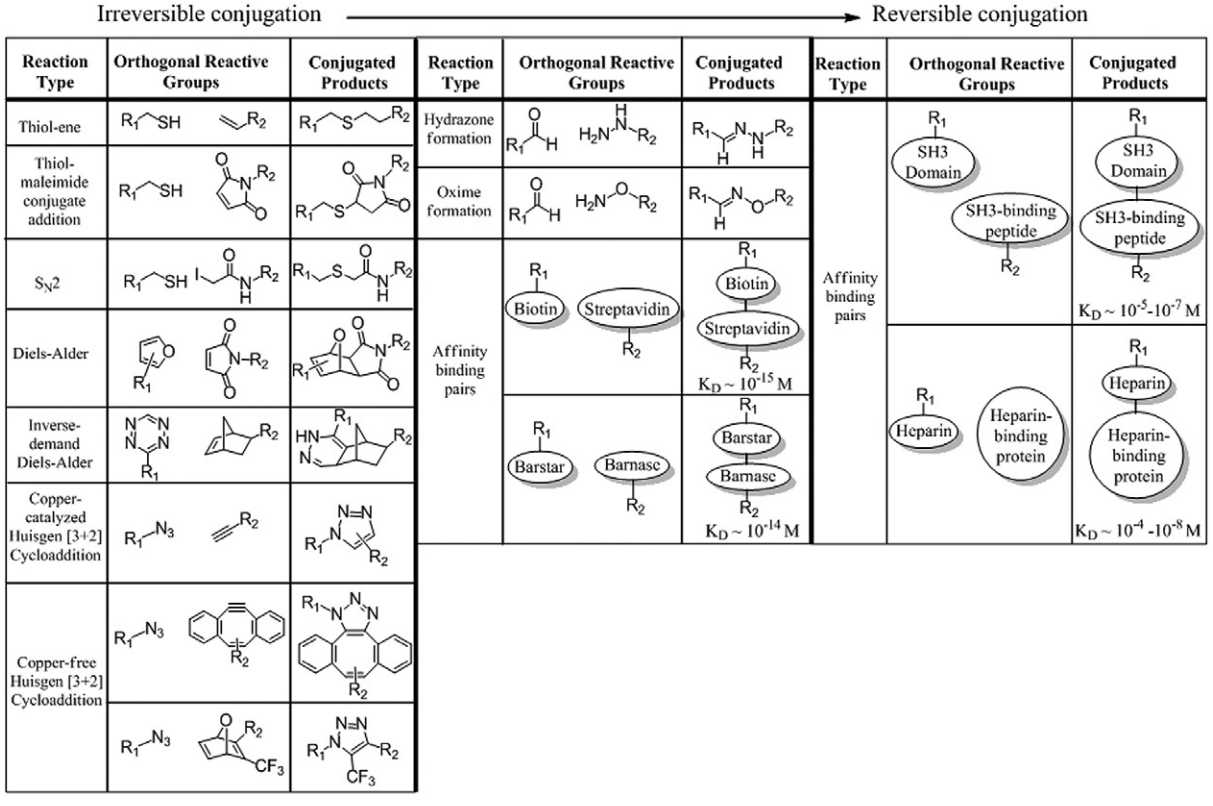

FIGURE 1.2 Typical orthogonal chemical reactions enable biomaterials to be synthesized with defined chemical and physical properties. Shown are a series of orthogonal *click* reactions which form covalent, irreversible (left panel) [27, 65, 79, 101–106] to near-covalent (middle panel) [63, 65, 107] to reversible (right panel) [52, 69, 70, 108, 109] bonds. For peptide or protein immobilization, this approach results in biomaterials that promote specific cellular responses, such as adhesion, proliferation, migration, and/or differentiation, depending on the biomolecule immobilized.

1.2.1 Synthetic Peptides Derived from Fibronectin, Laminin, and Collagen

The fibronectin-derived RGD peptide sequence is among the most studied peptide sequences for cell adhesion, and has been reviewed extensively [17,24,25]. Fibronectin is a ubiquitous protein that binds to different integrin receptors and promotes cell adhesion and cell survival. Immobilizing this sequence to biomaterials using bond-forming chemistries such as 1-ethyl-3-dimethylaminopropylcarbodiimide and N-hydroxysuccinimide (EDC/NHS) is problematic because the carboxylate-containing aspartic acid (D) participates in a competing reaction with the C-terminal carboxylate, thereby complicating the orientation of the sequence immobilized [26]. Bio-orthogonal conjugation chemistries have been used to overcome this problem and have resulted in effective adhesion for a variety of cell types to different types of modified biomaterials (Table 1.1) [17,27,28].

Derivatives of the linear RGD sequence have been synthesized in efforts to increase its binding to integrin receptors. Studies show that the RGD sequence in native fibronectin resides at the tip of a loop, which provides it with structural rigidity and a favorable conformation for integrin binding [29,30]. These structural characteristics have inspired the synthesis of cyclic RGD sequences [31,32]. Synthetic cyclic RGD peptides provide comparable conformational characteristics to facilitate integrin binding, and their conjugation to biomaterials have shown greater bioactivity compared with linear RGD sequences [33,34]. For example, cyclic RGD was recently conjugated to poly(4-methylpent-1-ene) (TPX) membranes for use as artificial lung supports. Endothelial cells cultured on this material showed significant cell adhesion, which is important for hemostasis use in these devices [35].

Another consideration for improved integrin interaction with immobilized RGD peptides is the distance between the peptide and the polymer backbone. A peptide that is bound too close to the polymer backbone may be hindered by steric interactions to efficiently bind with the receptors. Wilson *et al.* recently reported RGD peptides with a PEG linker containing greater than 27 ethylene oxide, repeat units showed significant adhesion of telomerase-immortalized human corneal epithelial cells (hTCEpi) [36].

Another ECM protein that has been extensively studied is laminin. Similar to fibronectin, laminin plays important roles in the ECM such as facilitating cell adhesion, differentiation, and migration. The most widely studied synthetic peptides for laminin are YIGSR [37] and IKVAV [38]. YIGSR has been shown to promote cell adhesion to the laminin-binding receptors, while IKVAV has been shown to promote primarily adhesion and neurite outgrowth of dorsal root ganglia (DRGs) [39], as well as differentiation of neural progenitor cells (NPCs) [40].

Collagens are another important class of proteins found in the ECM. Collagens provide structural support and also interact with receptors to mediate cell adhesion, migration, and proliferation [41,42]. The general structure of collagen consists of a triple helix, formed by three polypeptide strands, which can further assemble to supramolecular structures such as planar sheet-like networks, fibrils, and fibers [42]. There are 28 isoforms of collagen, with types I and IV being the most predominant in the ECM. Early collagen-mimetic synthetic sequences included the repeating

TABLE 1.1 Biomimetic Peptides of Common ECM Proteins and Methods of Immobilization

ECM molecule	Synthetic peptide mimic	Polymer	Method of peptide conjugation (polymer–peptide)	Bioactivity	Reference
Fibronectin	RGD	Gellan gum	Furan–maleimide	Increased neural stem/progenitor cell viability and adhesion	[27]
		Methyl cellulose	Thiol–maleimide	Increased oligodendrocyte differentiation from NSPCs when also treated with PDGF	[65]
		Agarose	Thiol–maleimide	Increased cell adhesion of NSPCs	[28]
		PEG	Thiol–acrylate	Increased endothelial cell adhesion and migration	[110]
			Cu (I) catalyzed azide–alkyne	Increased fibroblast viability and adhesion	[104]
			Oxyamine–ketone	Increased MSC viability and adhesion	[111]
			Tetrazine–norbornene	Increased MSC viability and adhesion	[103]
		Hyaluronan	Thiol–acrylate	Increased fibroblast viability and adhesion	[112]
		Elastin-mimetic polypeptide	tetrakis(hydroxymethyl) phosphonium chloride (THPC)	Increased dorsal root ganglia neurite outgrowth	[113]
	Cyclic RGD	PEG diacrylate	NHS–amine	Increased endothelial cell adhesion	[33]
		Elastin-mimetic polypeptide	Activated carboxylic acid (PyBOP)—amine	Osteoblast adhesion	[114]
		poly(4-methylpent-1-ene) (TPX)	Inverse electron demand Diels–Alder	Increased endothelial cell adhesion	[35]

Laminin	YIGSR	PEG monoacrylate	NHS–amine	Increased β-cell viability	[115]
		Dextran	Acrylate–thiol	Increased DRG neurite outgrowth	[39]
	IKVAV	PEG monoacrylate	NHS–amine	Increased β-cell viability	[115]
		Dextran	Acrylate–thiol	Increased DRG neurite outgrowth	[39]
Collagen	GFOGER	PEG	Acrylate–thiol	Increase chondrogenic differentiation	[44]
Antithrombin III	K(βA)FAKLAARLYRKA	Fibrin	transglutaminase factor XIIIa	Binding to NGF to promoted neurite outgrowth	[108]
Vitronectin	CGKKQRFRHRNRKG	Polyacrylamide	Maleimide–thiol	Maintained pluripotency of human ES cells	[55]
Human natural killer cells (HNKCs)	FLHTRLFV	Collagen	EDC–amine	Motor neuron neurite outgrowth	[60]
Polysialic acid	SSVTAWTTG	Collagen	EDC–amine	Motor neuron neurite outgrowth and Schwann cell neurite extension	[60]

tripeptide unit (Gly-X-Y), where X and Y were pre-dominantly conformationally rigid prolines to facilitate the formation of a triple helix. Subsequent work by Farndale and coworkers showed that the synthetic sequence (GFOGER, where O is hydroxyproline) derived from collagen I has high affinity for the $\alpha_2\beta_1$ integrin. Garcia *et al.* have also synthesized a peptide with the GFOGER hexapeptide flanked with the triple helical sequence $(GPP)_5$ to promote the formation of the triple helix [43]. On conjugating to various surfaces, HT1080 cells showed dose-dependent cell adhesion, and MC3T3-E1 cells showed vinculin staining, which suggests focal adhesion through integrin binding. Conjugation of a similar peptide to PEG resulted in increased chondrogenic differentiation of human mesenchymal stem cells compared with cells cultured in controls of PEG alone [44].

1.2.2 Carbohydrate-Binding Peptides

Carbohydrates play a significant role in cell recognition and binding. The chemical structures of carbohydrate complexes (glycans) are diverse and complex. They consist of numerous monosaccharide units (up to 200 total units) covalently bonded to each other linearly or as branched structures, with each structure providing a unique binding affinity to other molecules [45]. GAGs are a class of linear anionic polysaccharides that can be posttranslationally conjugated to proteins in the Golgi complex to form glycoproteins. Glycoproteins that are transported to the cell membrane function as transmembrane proteins, whereby the glycan is exposed to the extracellular space and participates in cellular recognition and protein binding [46].

Heparin is a GAG that is commonly found either in the ECM or conjugated to a transmembrane protein (proteoglycan). Heparin binds with high affinity to a variety of proteins such as antithrombin III (AT III) [47], bFGF [48], VEGF [49], and BMP-2 [50], and presents the proteins for enhanced bioactivity [51]. Thus, the conjugation of heparin to biomaterials is useful for applications that require interactions with heparin-binding proteins (HBPs). Sakiyama-Elbert and Hubbell reported that covalent conjugation of the AT III-derived sequence K(βA)FAKLAARLYRKA to fibrin matrices strongly bound to heparin [52]. A short peptide sequence (NQEQVSP) was also incorporated into the N-terminus to enable enzymatic peptide ligation to the fibrin hydrogel by transglutaminase factor XIIIa [53].

Another important role of transmembrane GAGs is to recognize chemical signals from the surrounding environment. Keissling *et al.* have discovered that the vitronectin-derived peptide sequence CGKKQRFRHRNRKG binds to GAGs expressed on the cell surface of human embryonic stem cells (hESCs), and can maintain their expression of pluripotent markers after 3 months [54]. Moreover, hESCs cultured on polyacrylamide hydrogels conjugated with this sequence both proliferated and maintained greater pluripotency than cells cultured on gels containing the integrin-binding sequence CRGDS [55].

1.2.3 Glycomimetic Peptides

As described earlier, carbohydrates play a significant role in cell recognition and binding. Efforts to study the interaction between glycans and cells using chemical

analogs have been limited by the inability to readily and efficiently chemically synthesize complex polysaccharides, which are challenging synthetic targets due to the multiple glycosylation steps, and the need to preserve the numerous carbohydrate stereocenters. While antibodies can be used to bind to carbohydrate receptors, their size and stability have limited large-scale use.

Interestingly, synthetic peptides have been discovered that mimic the chemical structures of several complex polysaccharides. These peptides occupy a similar chemical space as the parent polysaccharides, and therefore can bind to similar polysaccharide receptors. For example, a peptide sequence (FLHTRLFV) that mimics glycans found on the cell surface of human natural killer cells (HNKCs) was discovered using phage display and antibody-binding assays [56]. Motor neurons cultured in the presence of the HNKC glycomimetic peptide showed significantly longer neurite outgrowth compared with those cultured in the absence of HNKC-peptides [56]. Masand *et al.* recently conjugated this peptide to collagen hydrogels using EDC chemistry, and as demonstrated, these hydrogels also increased neurite outgrowth and length of motor neurons compared with cells cultured on collagen alone.

Another important glycan group is polysialic acid (PSA), which is naturally found conjugated to a variety of different transmembrane proteins including neural cell adhesion molecules (NCAMs). The PSA is hypothesized to be involved in cell migration of neural cells and cancer cells. Novel PSA-mimetic peptides have been discovered, and delivery of these PSA-mimetic peptides into the brain and spinal cord showed improved functional recovery and tissue regeneration in various injury models [57–59]. Masand *et al.* have also conjugated this PSA-mimetic peptide to collagen hydrogels, and demonstrated an increase in neurite length of cultured dorsal root ganglion and motor neurons, and increased Schwann cell proliferation compared with cells cultured on collagen alone [60]. However, a mixture of both PSA and HNKC peptides to collagen hydrogels yielded neither an additive effect for neurite outgrowth nor proliferation, emphasizing the importance of understanding the underlying mechanism for synergistic effects.

1.2.4 Growth Factors

Recently, larger molecules such as proteins and growth factors have been conjugated to biomaterials in a site-specific manner. Previous methods used nonspecific conjugation of large proteins to hydrogel scaffolds through amide linkage chemistry, such as EDC coupling. This approach is problematic due to the presence of multiple amines and carboxylates found in many proteins; random amide bond formation may decrease or even block protein activity. The limitation of nonspecific amidation has been overcome by exploiting site-specific modification, including protein modification to include *click* moieties discussed earlier [61], or by noncovalently incorporating proteins through high-affinity binding with complementary peptides/proteins immobilized to the hydrogel [62,63].

Various genetic modifications have enabled the site-specific incorporation of sequences and functional groups that can interact with bio-orthogonal partners. Protein biotinylation is a widely studied posttranslational modification and can be selectively incorporated into a protein that has been modified with the biotin-ligase

recognition sequence (GLNDIFEAQ*K*IEWHE) [64]. Biotin ligase selectively and covalently binds a biotin moiety to the primary amine of the lysine (K) residue in this sequence. The biotinylated protein is subsequently immobilized to streptavidin-containing biomaterials through high-affinity binding ($K_D \sim 10^{-15}$ M). Tam *et al.* recently reported a thiolated derivative of methylcellulose conjugated to maleimide–streptavidin, followed by immobilization of biotin-containing platelet-derived growth factor (PDGF) [65]. This material was shown to increase the differentiation of rat neural stem/progenitor cells into oligodendrocytes *in vitro*, and also promote functional and tissue repair in rat models of spinal cord injury [66].

Another method to immobilize proteins to biomaterials is to incorporate growth factor-binding domains derived from larger proteins such as fibronectin. The fibronectin domain FN III 12-14 was shown to have high affinity for several growth factors such as VEGF, PDGF-BB and BMP-2 [67]. Martino *et al.* demonstrated that incorporation of this domain into a fibrin hydrogel with these growth factors significantly increased cell proliferation and migration of endothelial cells (ECs), smooth muscle cells (SMCs) and mesenchymal stem cells (MSCs), respectively *in vitro*, as well as improved wound and bone tissue healing *in vivo* [68].

Growth factors have been incorporated into hydrogels through other modified polypeptide-based binding pairs. Stoller *et al.* reported that SH3-binding domains have variable binding affinities for short hydrophobic peptides [69]. Building on this work, Vulic *et al.* expressed SH3 and bFGF as a fusion protein and immobilized the complementary SH3 peptide-binding domain to methyl cellulose [70]. Using this approach, bFGF was noncovalently bound to hydrogels and the release rate of bFGF from the scaffold was tuned based on the SH3 protein–SH3 peptide dissociation constant. In a separate approach, Ehrbar *et al.* developed two fusion peptides: one containing the glutamine acceptor substrate (NQEQVSPL) (Gln) and a synthetic analog of Protein A (ZZ), the second containing interleukin-4 (IL-4) and the fragment crystallizable (Fc) region of immunoglobulin G (IgG) antibodies [71]. The Gln-ZZ construct was conjugated to PEG hydrogels via enzymatic ligation to lysine donors on the hydrogel backbone. High-affinity binding (4.8×10^{-8} M^{-1}) between Protein A and Fc led to the incorporation of IL4 into the PEG hydrogels. The activity of immobilized IL4 was preserved as evidenced by a cell-based fluorescent reporter.

Several additional bio-orthogonal partners have been investigated as a means to noncovalently control the extent and duration of growth factor presentation in hydrogels (Table 1.2). Potentially, any growth factor that can be stably expressed as a fusion protein can be adapted for one of these approaches.

1.3 SPATIAL ORIENTATION AND DYNAMIC DISPLAY

During tissue development, tissue repair, and many disease states, the composition of the ECM is dynamic [72]. Specifically, growth factors and adhesive molecules are often presented transiently and localized in specific locations or as gradients within the ECM. Therefore, the next major challenge for incorporating ECM-inspired molecules into hydrogels is to allow user-defined temporal and spatial control over the presentation of biomimetic cues discussed above [73].