

PART **I**

*ENGINEERING
BIO-INSPIRED MATERIAL
MICROENVIRONMENTS*

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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

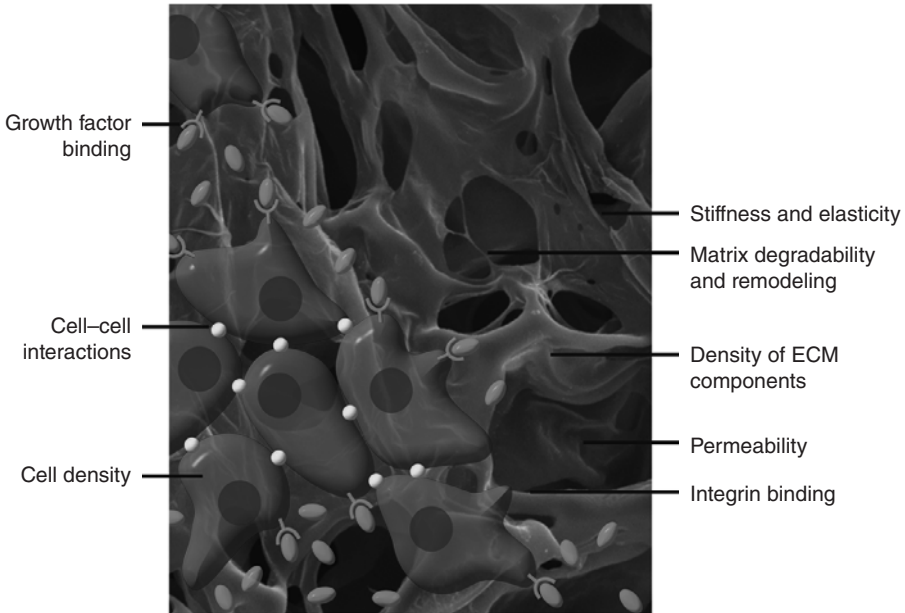


FIGURE 1.1 The complex 3-D cellular environment provides mechanical and biochemical signals that guide cell function. The components of the ECM dictate the stiffness of matrix and the types of cell–matrix interactions. The matrix composition determines the ease with which nutrients diffuse through tissues 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].

Irreversible conjugation		Reversible conjugation				
Reaction Type	Orthogonal Reactive Groups	Conjugated Products	Reaction Type	Orthogonal Reactive Groups	Conjugated Products	
Thiol-ene			Hydrazone formation			 $K_D \sim 10^{-5}-10^{-7} \text{ M}$
Thiol-maleimide conjugate addition			Oxime formation			
S_N2			Affinity binding pairs			 $K_D \sim 10^{-4}-10^{-8} \text{ M}$
Diels-Alder						
Inverse-demand Diels-Alder			Affinity binding pairs			$K_D \sim 10^{-15} \text{ M}$
Copper-catalyzed Huisgen [3+2] Cycloaddition						
Copper-free Huisgen [3+2] Cycloaddition			Affinity binding pairs			$K_D \sim 10^{-15} \text{ M}$

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]
			Oxamine-ketone	Increased MSC viability and adhesion	[111]
			Tetrazine-norbormene	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]
			NHS-amine	Increased endothelial cell adhesion	[33]
			Activated carboxylic acid (PyBOP)-amine	Osteoblast adhesion	[114]
			Inverse electron demand Diels-Alder	Increased endothelial cell adhesion	[35]
	Cyclic RGD	PEG diacrylate			
		Elastin-mimetic polypeptide			
		poly(4-methylpent-1-ene) (TPX)			

Laminin	YIGSR	PEG monoacrylate	NHS-amine	Increased β -cell viability	[115]
	IKVAV	Dextran	Acrylate-thiol	Increased DRG neurite outgrowth	[39]
	GFOGER	PEG monoacrylate	NHS-amine	Increased β -cell viability	[115]
Collagen		Dextran	Acrylate-thiol	Increased DRG neurite outgrowth	[39]
		PEG	Acrylate-thiol	Increase chondrogenic differentiation	[44]
Antithrombin III	K(β A)FAKLAARLYRKA	Fibrin	transglutaminase factor XIIIa	Binding to NGF to promoted neurite outgrowth	[108]
Vitronectin	CGKKQRFRRNRKKG	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(β A)FAKLAARLYRKA to fibrin matrices strongly bound to heparin [52]. A short peptide sequence (NQE₂QVSP) 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 CGKKQRFHRNRKG 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 (GLNDIFEAQKIEWHE) [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].

TABLE 1.2 Growth Factors Immobilized to Hydrogels Through Bio-Orthogonal Partner Binding

Growth factor	Polymer	Binding partners	Bioactivity	Reference
bFGF	Methyl cellulose	SH3 protein—SH3binding peptide	NSPC survival	[70]
	PEG	bFGF—bFGF-binding peptide	Promoted differentiation	[116]
	Agarose	Albumin—albumin-binding domain	Neurosphere assay	[80]
BMP-2	Fibrin	FN III 12-14 binding domain	Increased MSC migration	[68]
CNTF	Agarose	Biotin-streptavidin	Promoted differentiation of retinal cells	[63]
IFN	methacrylate	Biotin-streptavidin	Promoted differentiation to neurons	[117]
IL4	PEG	Fc—synthetic protein A	Activation of reporter	[71]
PDGF	Methyl cellulose	Biotin-streptavidin	Increased NSPC differentiation into oligodendrocytes, increased functional recovery in rats with spinal cord injury	[65, 66]
	Agarose	Thiol—maleimide	Increased NSPC differentiation into oligodendrocytes	[28]
	Fibrin	FN III 12-14 binding domain	Increased smooth muscle cell (SMC) and MSC proliferation and migration	[68]
SHH	Agarose	Barstar—barnase	Increased NSPC migration	[63]
VEGF	Fibrin	FN III 12-14 binding domain	Increased endothelial cell proliferation and migration	[68]
	Agarose	Thiol—maleimide	Increased EC and RSPC migration	[78]

1.3.1 Spatially Controlled Display

Incorporating biomimetic cues into engineered scaffolds is essential. As discussed above, significant progress has been made in immobilizing ECM-inspired molecules into hydrogels. The majority of examples incorporate biomolecules into hydrogels uniformly. Recently, photochemical patterning has emerged as a powerful approach to control the spatial immobilization of biomolecules within 3-D hydrogels [74–76].

The majority of *photopatterning* or *photolithography* has been focused on the immobilization of cell adhesive molecules, such as RGD and IKVAV, by directly conjugating these peptides to the hydrogel backbone using light-activated ligation

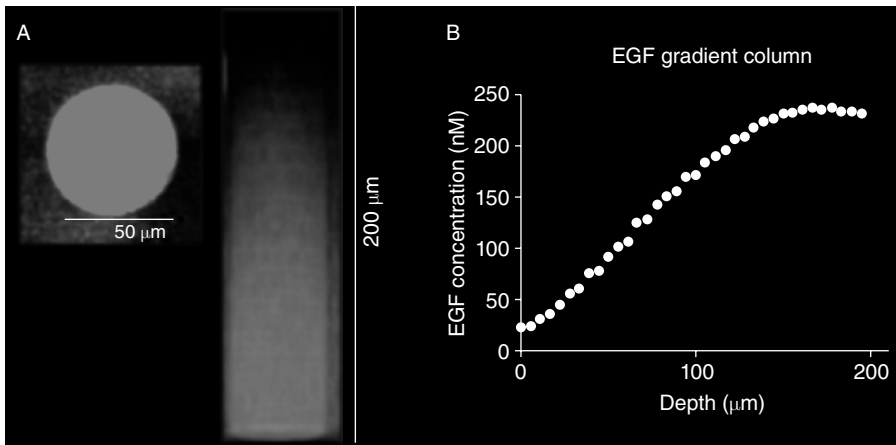


FIGURE 1.3 3-D photopatterning of EGF within a hyaluronic acid-PEG hydrogel. (A) Creation of a linear immobilized gradient of EGF. From the top of the hydrogel, the number of scans by the multiphoton laser are increased as it penetrates into the sample, corresponding to an increase in fluorescence intensity, and hence, an increase in protein immobilization. (B) The concentration of immobilized protein in the gradient was quantified by fluorescence intensity, showing a change in concentration from 25 nM at the top of the hydrogel to 250 nM at a depth of 150 μm in the hydrogel. EGF, epidermal growth factor. (Reproduced with permission from Owen, S.C., Fisher, S.A., Tam, R.Y., Nimmo, C.M., Shoichet, M.S. *Langmuir* **2013**. Copyright 2013 American Chemical Society.) (See insert for color representation of the figure.)

[78]. In this manner, Aizawa *et al.* patterned gradients of VEGF into agarose hydrogels and demonstrated that the immobilized proteins remained bioactive and effectively guided endothelial cell migration and tubulogenesis. Successful photopatterning has also been demonstrated in natural-synthetic hybrid hydrogels. For example, Owen *et al.* adapted coumarin-based photochemistry [63] into hyaluronic acid hydrogels to immobilize epidermal growth factor (EGF) gradients, Figure 1.3 [80]. Wylie *et al.* advanced photopatterning technology to simultaneously immobilize multiple proteins into hydrogels through bio-orthogonal paired *click* reactions [63,81]. The authors first conjugated one-half of the bio-orthogonal pair (barnase or streptavidin) directly to the hydrogel backbone. The complementary binding partner was coexpressed or ligated to a functional protein, in this case CNTF-biotin and sonic hedgehog (SHH)-barstar, and then mixed in the hydrogels where they bound specifically with their immobilized partners. Using this approach, both of the functional proteins were incorporated in spatially defined gradients within the hydrogels. It was further demonstrated that cultured neural progenitor cells responded to both CNTF and SHH gradients.

Photochemistry can also be exploited to remove biomimetic cues. Anseth and coworkers incorporated photodegradable cross-linking technology into PEG hydrogels in order to selectively degrade the scaffold [82] or remove adhesive biomolecules [83]. Significantly, the bio-*clip* removal of biomimetic peptides was

recently combined with photopatterning technology to allow reversible presentation [84]. Recently, Kasko and coworkers have significantly expanded the number of different photocleavable linkers to facilitate conjugation of bioactive compounds [85].

Additional techniques are being investigated to control the immobilization of biomolecules in defined regions of interest or as gradients. For example, Turturro *et al.* employed perfusion-based frontal photopolymerization to create biofunctional gradients of RGD in matrix metalloproteinase (MMP)-sensitive hydrogels [86]; Wan *et al.* used electrical currents in conducting hydrogels to induce gradients of protein deposition and subsequent cell density [87,88].

1.3.2 Stimuli-Sensitive Dynamic Display

A second approach to control the display of biomimetic cues is to utilize hydrogels, which are sensitive to external stimuli such as temperature, pH, or enzymes. A number of stimuli-sensitive hydrogels have been developed and are reviewed extensively elsewhere [88–91]. For tissue engineering applications, the ability to control the presentation and the removal of specific cell adhesion proteins or growth factors may permit the controlled proliferation and differentiation of stem and progenitor cells.

Using enzymatic cleavage to release appended growth factors [62], Zisch *et al.* conjugated VEGF to PEG hydrogels by an MMP-cleavable synthetic peptide linker [92]. Similarly, Lutolf *et al.* utilized similar PEG hydrogels modified with pendant recombinant human bone morphogenetic protein-2 (rhBMP-2) also cleaved by MMP [93]. In both of these approaches, the growth factors are presented on the hydrogel surface but only released upon local cellular demand.

Many recent endeavors are more specifically focused on utilizing external stimuli to control the exposure of biomolecules. Okano and coworkers pioneered the use of temperature-sensitive poly(N-isopropylacrylamide) (poly(NIPAAm)) hydrogels to engineer *cell sheets* from several cell types [94–96]. Recently, Okano's group developed a modified version of poly(NIPAAm) by covalently tethering heparin onto poly(N-isopropylacrylamide-co-2-carboxyisopropylacrylamide) hydrogel surfaces. As discussed, heparin possesses an affinity for a number of growth factors, including VEGF and bFGF, and is important in stabilizing these proteins. As such, the heparin-functionalized hydrogels readily bind these growth factors on gels at 37°C and release the growth factors upon swelling of poly(NIPAAm-co-CIPAAm) chains at 20°C. Okano's group first incubated bFGF in heparin-modified gels and then cultured NIH/3T3 cells on the surface, demonstrating that heparin-bound bFGF enhanced growth factor-specific cell attachment. In a separate study, Tekin *et al.* used poly(NIPAAm) hydrogels to promote the formation and retrieval of stable cell aggregates for various applications [97].

Additional stimuli-sensitive hydrogels are in the early stages of development for tissue engineering applications. For example, Maynard and coworkers synthesized a novel temperature sensitive hydrogel based on poly(triethylene glycol methacrylate) (poly(TEGMA)) [98]. Significantly, the spatial deposition of these hydrogels can be controlled using e-beam radiation lithography. Further adaption of

these hydrogels with biomimetic factors could provide user control over multiple cell-instructive cues.

1.4 FUTURE PERSPECTIVES

The ability to systematically re-create the 3-D microenvironment that defines cellular function and organization has profound implications for applications such as tissue regeneration strategies and understanding disease progression, including elucidating complex cellular mechanisms and functions. Current technologies such as immunochemistry and phage display have provided high-throughput methods to discover molecules that are involved in cellular interactions and techniques to develop synthetic peptide analogs. These strategies can now be combined with high-content information achieved with biomimetic strategies of the cell niche.

The combination of *bio-click* and *bio-clip* photopatterning are particularly exciting as they enable well-defined chemical cues that both guide and respond to cell fate. Although not discussed in this chapter, the mechanical properties of the ECM also influence cell fate, in concert with the chemical and physical properties. There is evidence that both the underlying elasticity of the ECM [99] and the tethering of cells to the ECM [100] play significant roles in stem cell growth and differentiation. As such, modular hydrogels, with independent user control over physical and biochemical properties, will facilitate our ability to understand and guide cell behavior. The cell itself has significant impact over its environment, and thus the culture of multiple cell types at different cell densities are additional considerations in the design of a bioengineered 3-D hydrogel matrix.

Abbreviations

AT III	Antithrombin III
bFGF	Basic fibroblast growth factor
BMP-2	Bone morphogenetic protein 2
CNTF	Ciliary neurotrophic factor
DRG	Dorsal root ganglion
ECM	Extracellular matrix
ECs	Endothelial cells
EGF	Epidermal growth factor
ES cells	Embryonic stem cells
HBP	Heparin-binding protein
HNKCs	Human natural killer cells
HT1080	Fibrosarcoma cell line
IFN	Interferon
IgG	immunoglobulin G
IKVAV	Synthetic peptide derived from the α -1 chain of laminin
IL-4	Interleukin-4
MC3T3-E1	Osteoblast precursor cell line
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells

NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NSPCs	Neural stem/progenitor cells
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PSA	Polysialic
RGD	(Arginine–glycine–aspartic acid): synthetic peptide derived from fibronectin
rhBMP-2	Recombinant human bone morphogenetic protein-2
RSPCs	Retinal stem/progenitor cells
SHH	Sonic hedgehog
VEGF	Vascular endothelial growth factor
YIGSR	(Tyr-ile-gly-ser-arg): synthetic peptide derived from β -1 chain of laminin

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