TISSUE ENGINEERING: Part B
Volume 14, Number 4, 2008

DOI: 10.1089/ten.teb.2008.0304

Strategies and Applications for Incorporating Physical and Chemical Signal Gradients in Tissue Engineering

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From embryonic development to wound repair, concentration gradients of bioactive signaling molecules guide tissue formation and regeneration. Moreover, gradients in cellular and extracellular architecture as well as in mechanical properties are readily apparent in native tissues. Perhaps tissue engineers can take a cue from nature in attempting to regenerate tissues by incorporating gradients into engineering design strategies. Indeed, gradient-based approaches are an emerging trend in tissue engineering, standing in contrast to traditional approaches of homogeneous delivery of cells and/or growth factors using isotropic scaffolds. Gradients in tissue engineering lie at the intersection of three major paradigms in the field—biomimetic, interfacial, and functional tissue engineering—by combining physical (via biomaterial design) and chemical (with growth/differentiation factors and cell adhesion molecules) signal delivery to achieve a continuous transition in both structure and function. This review consolidates several key methodologies to generate gradients, some of which have never been employed in a tissue engineering application, and discusses strategies for incorporating these methods into tissue engineering and implant design. A key finding of this review was that two-dimensional physicochemical gradient substrates, which serve as excellent high-throughput screening tools for optimizing desired biomaterial properties, can be enhanced in the future by transitioning from two dimensions to three dimensions, which would enable studies of cell–protein–biomaterial interactions in a more native tissue–like environment. In addition, biomimetic tissue regeneration via combined delivery of graded physical and chemical signals appears to be a promising strategy for the regeneration of heterogeneous tissues and tissue interfaces. In the future, *in vivo* applications will shed more light on the performance of gradient-based mechanical integrity and signal delivery strategies compared to traditional tissue engineering approaches.

Introduction

From rocks[1] to squid beaks,[2] nature is rich with gradients. Chemical signal gradients drive embryonic development, whereas gradients in cellular–extracellular architecture exist throughout the human body, within tissues and at tissue interfaces, to satisfy spatially diverse functional needs. In order to engineer complex tissues, gradient-based strategies can be incorporated into tissue engineering.

In this review, we discuss gradient-generation methodologies, some of which have been applied in tissue engineering investigations, while many others hold potential to be incorporated into biomaterial design or for spatially controlled delivery of bioactive factors. From the perspective of traditional tissue engineering, gradient-based substrates have provided a quick single-experiment route to optimize biomaterial characteristics without introducing the experimental artifacts generated due to discrete substrate preparations, thus serving as a tool for high-throughput screening of biomaterials. More importantly, chemical and/or physical gradients can be directly incorporated into the design of biomaterials to engineer heterogeneous tissues and tissue interfaces. Combined with spatially and temporally controlled delivery of exogenous bioactive factors, the gradient-based tissue engineering approaches may provide an effective scheme to engineer tissues and organs. The known sensitivity of cells to various physical and chemical stimuli, which cause cell migration (or "-taxis") (Table 1),[3,4] has yet to be fully utilized in tissue engineering. Integration of spatially controlled signal gradients with tissue engineering may lead to dynamic cellular machineries that could enhance such cell-based therapies.

Gradient-based devices and strategies are employed in practice in a plethora of fields commercially, such as in electrophoresis,[5] dielectrophoresis,[6] chromatography (e.g., gradient elution generators in liquid chromatography), the aerospace industry,[7] and the discovery of drugs, materials, and catalysts.[8] Here, we concisely present gradient-generation techniques relevant to the tissue engineering community, many of which have never before been employed

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Table 1. A Summary of Various Forms of Physical and Chemical Stimuli, Gradients of Which Are Known to Provide a Control Over Cell Movement or "Taxis" (Adapted from Lo *et al.*[3] and Haga *et al.*[4])

Stimulus		
Type	*Form*	*Form of "-taxis"*
Chemical	Dissolved form	Chemotaxis
	Immobilized form	Haptotaxis
Physical	Substrate rigidity	Durotaxis/ mechanotaxis
	Electrostatic potential	Galvanotaxis
	Light intensity	Phototaxis
	Gravitational potential	Geotaxis
	Extracellular tension	Tensotaxis

in tissue engineering applications, and emphasize specific possible future roles of mechanical and signal gradients in next-generation tissue engineering strategies.

Biomaterial-Based "Physical" Signal Gradients

Tissues consist of cells and extracellular matrix (ECM), and differ with each other in type, content, and organization of the constituent cells and ECM components. In tissue engineering, these differences must be considered before selecting biomaterials and designing scaffolds for a specific application. In addition, findings of several studies probing cell–biomaterial interactions suggest that biomaterials at the micro/nanoscale may also act as a "physical" signal that may affect cell behavior, such as adhesion, spreading, motility, survival, and differentiation. The following is a discussion of the importance of gradient-based strategies that (1) can be utilized in the identification of optimal scaffold design parameters for traditional homogeneous scaffolds, and (2) can directly be incorporated as physical and chemical signals in the heterogeneous scaffold design for specific tissue engineering applications. We also briefly discuss the use of gradient-based approaches in the design of implants used for orthopedic and orthodontic repair procedures.

Polymeric Materials

In this section, we discuss the strategies that have been applied for creating polymer-based gradients, and also emphasize the possible applications of these gradients from a tissue engineering perspective. Among several scaffold design parameters, we have considered a select few: pore size, porosity, material stiffness, and surface physicochemical characteristics, which are of high interest.

Pore-size/porosity gradients

Among several scaffold design parameters, pore size and porosity bear prime importance. Pore size is known to affect cellular affinity and viability by influencing cellular movement, binding and spreading, intracellular signaling, and transport of nutrients and metabolites.[9] Porosity governs the maximum possible accommodation of cell mass in the scaffold[10]; however, high porosity values often compromise mechanical properties of the scaffold.[11] From an application standpoint, pore size and porosity also affect neovascularization *in vivo*.[10] In addition, the scaffold architecture also requires consideration. An interconnected pore network is desired to minimize dead volume, and tortuosity of the network requires attention from a mass transport perspective.[12] Pore shapes may also critically affect the cellular organization in the scaffolds,[13,14] as substantiated by several studies based on the contact guidance theory. The importance of scaffold architecture in tissue engineering is increasingly being realized, which has resulted in a change in trend in the designs of scaffolds, from isotropic scaffolds to heterogeneous and anisotropic "biomimetic" scaffolds, with the goal being to mimic the organization of the cells (such as alignment or clustering) and/or the ECM of the tissue under consideration.

For designing isotropic scaffolds, the required range of optimal pore sizes depends on the intended tissue engineering application (i.e., the type(s) of cells/tissues that will interact with the scaffold).[9,10,15] In this regard, scaffolds containing a pore-size gradient provide a rapid screening tool to probe *in vitro* cell–scaffold or *in vivo* tissue–scaffold interactions, as demonstrated earlier,[9] which may help in the identification/validation of the optimal pore sizes. Such graded porous scaffolds can be instrumental in the engineering of specific tissues that possess highly zonal architecture or interfacial tissues, consisting of organized multiple cell layers and extracellular environments. So far, only a few such reports exist, for example, where native zonal organization of the cartilage was accounted for in the scaffold design by utilizing an anisotropic gradient-based pore architecture,[16] or where heterogeneous organization of an osteochondral tissue was considered by including gradients in material composition (and thus, mechanical properties) and pore size in the scaffold design.[17] In one study, a prototype of graded bone implant with spatially varying pore size and porosity (in the radial direction) was created.[18] Gradients in material composition at the transition region may also reduce or eliminate the problem of delamination that is commonly observed in similar biphasic scaffold designs.[17] Scaffolds exhibiting pore-size and porosity gradients can also provide control over cell migration, where the migration can be restricted in the direction of decreasing pore sizes by appropriately selecting the pore sizes,[19] or can be facilitated in the direction of increasing porosity to guide tissue ingrowth.[20]

Application of pore-size gradients is common in pore gradient gel electrophoresis.[21] Due to its purpose, the size of the pores in such gels allows permeability at the protein-size level, not at the cellular level. To create continuous gradients in pore size as well as porosity in macroporous scaffolds (permitting movement at the cellular level), techniques that have been developed include a centrifugation–heat-sintering method,[9] a combination of melt pressing and porogen leaching,[18] a centrifugation–freeze drying method,[19] and a phase separation–freeze drying method.[22,23] Using a three-dimensional (3D) printing technique in combination with porogen leaching, scaffolds having gradients in porosity with near-uniform pore sizes were created.[17,20] Solid free-form fabrication methods would in general be quite amenable to creating pore-size and other structure-based gradients. A novel 3D fiber deposition technique was utilized in creating scaffolds that contained a gradient in pore size with a uniform porosity.[16] Table 2 summarizes the specifics of the aforementioned studies, which provide routes to creating 3D scaffolds with a pore-size and/or porosity gradient(s).

Table 2. Fabrication Techniques Used to Prepare Continuous Macroscopic Gradients in Porosity and/or Pore Size in 3D Scaffolds

Gradient type	*Fabrication method*	*Geometry/gradient direction*	*Materials used*[a]	*Primary gradient shape-controlling process parameter(s)*[b]	*Application*	*References*
	Centrifugation–heat sintering	Cylindrical/axial	PCL-pluronic F127	f(Centrifugal speed), e.g., pore sizes ~88–405 μm and porosities ~80–94% at 3000 rpm	To investigate the cell–tissue interaction with the scaffold in order to optimize the pore size	9
	Centrifugation–freeze drying	Tubular/radial	Collagen–GAG	f(Centrifugal speed, time), e.g., pore sizes <5 μm (at the wall) to ~20 μm (near the lumen) at 30,000 rpm for 15-min spinning time	"Mold-less" creation of tubular scaffolds; to study myofibroblast migration during peripheral nerve regeneration	19
Pore-size and porosity gradient	Phase separation (by temperature gradient–driven cryogenic treatment)—freeze drying	Cuboid shaped/ normal (in the direction of heat transfer)	Gelatin hydrogel (chemically crosslinked)	f(Cooling rate, temperature gradient), e.g., pore sizes 20–30 to 330 μm and porosities 61–82% for 30°C temperature gradient at 0.15°C/min cooling rate with 10% (w/v) gelatin	Gelatin scaffolds for tissue engineering with controllable pore size, pore geometry, and porosity	22
	Solid state polymerization–melt pressing–porogen leaching	Cylindrical/radial or axial	PGA	f(Porogen content and porogen size), e.g., macroscopic pore size ~300 μm and microscopic pore size 0.3 μm	Scaffold that mimics natural bone	18
Porosity and material composition gradient	3D printing (TheriForm™)–porogen leaching	A disc on a cloverleaf/axial	PLGA–PLA–TCP	Programmable porosity gradient: f(axial porogen content) (55–90%), pore size: f(porogen particle diameter) 106–150 μm	Scaffold for osteochondral defect repair	17
Porosity gradient	3D printing (TheriForm™)–porogen leaching	Disc/axial	PLGA with 20% β-TCP	Programmable porosity gradient: f(axial porogen content), pore size: f(porogen particle diameter) 125–150 μm	A composite material for bone defect repair	20
Pore-size gradient	3D fiber deposition technique	Disc/axial	PEGT–PBT	Programmable pore-size gradient: f(fiber deposition pattern) (200–1650 μm)	Scaffolds with anisotropic pore architecture to engineer cartilage with native zonal organization	16

[a]PCL: poly(caprolactone); GAG: glycosaminoglycan; PGA: poly(glycolic acid); PLGA: poly(lactic-co-glycolic acid); PLA: poly(L-lactic acid); TCP: tricalcium phosphate; PEGT: poly(ethylene glycol)-terephthalate; PBT: poly(butylene terephthalate).

[b]Primary "gradient-controlling" parameter is the parameter investigated and found to have a profound effect over the gradient profile; the influencing parameters are reported as: f(process-dependent parameter(s)).

Substrate stiffness gradients

Substrate stiffness affects cellular adhesion, spreading, motility, survival, and differentiation (for details, see reviews by Discher *et al*.,[24] Schwarz,[25] and Li *et al*.[26]). Research with fibroblasts, epithelial cells, and smooth muscle cells has demonstrated that cell contact with the substrate diminishes on increasingly softer substrates,[27,28] and cells migrate from softer regions to stiffer regions when exposed to a gradient in substrate stiffness,[3,29–32] a phenomenon termed "durotaxis" or "mechanotaxis" (Table 1). A recent review by Georges and Janmey[33] summarizes findings with various cell types (e.g., endothelial cells, neurons, and hepatocytes) that provide corroborating evidence for the importance of substrate stiffness as a physical signal for cells. An "effective" stiffness range, however, varies between cell types and leads to cell-specific responses.[33] For example, fibroblasts placed on various soft polyacrylamide substrates (shear modulus: <1.6 kPa[34]; Young's modulus [AFM]: 14 kPa,[3] ~ 2.7 kPa[35]; Young's modulus [bulk]: 1.8 kPa,[31] ~ 4.4 kPa[35]) display a change in their cytoskeletons (destabilized focal adhesions, and loss in actin filament or "stress fiber" expression, resulting in a rounded cellular morphology) compared to corresponding stiffer substrates (shear modulus: >3.6 kPa[34]; Young's modulus [AFM]: 30 kPa,[3] ~ 7.7 kPa[35]; Young's modulus [bulk]: 34 kPa,[31] 12.4 kPa[35]), where these cells adapt a flat morphology. Neurons preferentially branch on soft substrates (50 Pa) compared to stiff surfaces (550 Pa), while the range of stiffness that is relevant to neurons may not affect fibroblasts.[35,36] Smooth muscle cells prefer moderately stiff surfaces ($E \sim 8$–10 kPa) to display tissue-like actomyosin patterns and cellular spreading areas compared to softer or stiffer substrates.[37,38] Also, the response of the cells to the physical cues is generally not isolated from the presence of cell-to-cell interaction and haptotactic (surface-bound) cues.[34,39] These pioneering studies have proven the importance of substrate stiffness for cells; however, many of these studies utilized homogeneous or step-gradient substrate preparations possessing randomly selected stiffnesses, which provided little information regarding the "threshold values" of stiffness that critically alter the behavior (e.g., morphology, clustering, and apoptosis) of a particular type of cell. In this regard, use of substrates containing continuous gradients with a large range in stiffness may provide a means to systematically study the cell response to substrate stiffness and accurately identify the threshold substrate properties critical to cellular behavior.[30] Continuous gradient surfaces may also facilitate the understanding of molecular mechanisms of cellular dependence on substrate stiffness.

Strategies to design tissue-engineered replacements require judicious selection of the biomaterials with one of the goals being to satisfy the biomechanical requirements of musculoskeletal tissues. Material selection and scaffold design become particularly important when developing heterogeneous tissues or tissue interfaces that are biomechanically anisotropic and endowed with gradients in stiffness by nature, such as an osteochondral tissue, the intervertebral disc, skin layers, blood vessel walls, and so on. Importantly, some investigations suggest that a tissue-like *in vitro* growth of cells (such as tissue-like striated actomyosin patterns and cellular spreading areas, as in the case of smooth muscle cells, or elevated branching, as in the case of neurons) requires substrate mechanical properties to be close to that of the ECM of the native tissue.[36–38] While these findings certainly require validation of the relationship between physiologically relevant cellular morphology and cell functions,[30] the implication of these findings could be relevant to tissue engineers because it may provide justification to the current criteria of biomaterial selection and design. It may further implicate the need to design heterogeneous constructs for interfacial tissue regeneration, containing built-in continuous 3D gradients in stiffness imparted by the specific biomaterials, which may not only lead to a mechanically robust tissue-engineered replacements, but may also prove useful in cell-mediated tissue repair. In addition, *in vivo* cellular infiltration in the biomaterial from the surrounding tissue can be optimized by suitably selecting material properties that may promote durotaxis toward the tissue-engineered replacement. Also, due to the diversity in the ranges of effective stiffness to which a cell responds (as mentioned earlier), gradient substrates hold potential to be utilized in durotaxis-driven cell separation and sorting applications commercially.

Two-dimensional (2D) substrates containing both a step-transition and a continuous gradient in stiffness have been made in the past. Using soft lithography, poly(dimethylsiloxane) substrates containing near-absolute step gradients in stiffness were fabricated, and utilized to study durotaxis and micropatterning of cells.[31] In some studies (including the breakthrough work by Wang and colleagues), a diffusion-based photopolymerization approach was utilized to create substrates that contained a sharp transition in material stiffness with a thin (extending to several microns) stiffness-gradient region.[3,31,32] To fabricate substrates containing controlled continuous stiffness gradients at macro- and microscales, controlled photopolymerization processes have been utilized in the past, where gradients were generated by controlled photoexposure (using a gradient photomask[29,40] or by varying photoexposure time[41]) or by precisely altering the crosslinker concentration using a microfluidic device.[30,37] In many of these early investigations, polyacrylamide gels served as tools to create step and continuous compliance substrates. Various other materials that have similarly been used to create substrates with continuous stiffness gradients include a two-component dimethacrylate blend,[41] styrenated gelatin,[32] and PEG-diacrylate.[42]

In summary, the utility of stiffness gradient surfaces has so far been realized in studying durotaxis phenomena, micropatterning of cells, and high-throughput screening of materials. Probing other possible cell–biomaterial combinations using continuous stiffness gradient surfaces (as a screening tool) may provide useful information to the tissue engineering community. Stiffness gradient constructs may also be useful in interfacial tissue regeneration; however, it will require a transition from 2D surfaces to 3D environments. The challenge lies in gathering further knowledge and combining it toward designing "active" optimal bioengineered constructs.

Surface gradients in physicochemical characteristics

Surface physicochemical characteristics of a biomaterial (e.g., wettability [hydrophilicity], roughness, crystallinity, charge, and functionality) may critically influence the host response to an engineered replacement by affecting protein–biomaterial and cell–biomaterial interactions *in vivo*, directly

or indirectly (as reviewed by Ruardy *et al*.[43]). Tailoring the surface features in a user-specified manner can provide spatial control over relevant phenomena, such as protein adsorption and cellular adhesion, proliferation, and morphology, selectively or nonselectively. For example, cell repellant surface functional groups can be utilized and/or surface wettability can be controlled to diminish scar tissue formation or reduce platelet adhesion and activation.[44] For a different application, the same functional groups can be exploited to immobilize nanospheres that may alter cellular spreading and morphology, or to immobilize bioactive factors of interest over the surface (for example, by utilizing surface carboxyl or amine functionalities) to impart other desired features.[45,46] For tissue engineering advancements, surface physicochemical gradients continue to serve as a fast-screening tool to identify the threshold regimes in physicochemical characteristics to optimize biomaterial properties (e.g., improved biomaterial coatings, and application-specific protein and cell interaction with the biomaterial).[47] In addition, patterned and gradient substrates can be utilized to alter site-specific biomaterial properties.[44] In the following paragraphs, we highlight some of the well-developed surface chemistry gradient-generation methodologies (summarized in Table 3). These continuous surface chemistry gradient-generation methodologies can be divided into two major groups, as described by Genzer and Bhat[48]: (1) top-down (involving modification of a substrate via physical and/or chemical treatments) or (2) bottom-up (involving deposition onto the substrate). For a more detailed overview, we recommend recent review articles by Kim *et al*.[47] and Genzer and Bhat,[48] where surface chemistry gradients and characterization techniques have been comprehensively discussed.

Wettability gradient surfaces have been produced using surface etching techniques, such as UV-ozonolysis (UVO),[49–51] radio-frequency gas plasma discharge,[52–55] power-graded corona discharge treatment,[56–63] or a combination (UVO-plasma).[64] Peroxide initiators generated during the etching process lead to oxidation of the substrate surface and induce oxygen-based hydrophilic functionalities (such as −OH, −COOH, and −COOR). By varying a process-specific parameter continually (such as exposure intensity, exposure time, and power), a surface with a continuously increasing surface energy and wettability results. Deposition of monomer(s) during the induction or graft copolymerization following the induction of these functionalities results in polymer-templated substrates, where gradients can be produced by monomer composition and/or by varying a process-specific parameter in a controlled manner. Examples include UV grafting,[41,46,65–67] grafting following power-graded corona discharge,[68–73] and plasma deposition[74–77] (Table 3). With a proper choice of monomers, the chargeable functional group gradients were created to investigate the effect of charge density over cell behavior.[70,71] In addition to the functional group density and wettability gradients, a gradient in nanoscale thickness of the substrate usually develops following the graft copolymerization, where the grafted polymer shows a transition from a loosely packed "mushroom" regime to a densely packed "brush" regime.[78] Other creative methods utilized to fabricate wettability gradient surfaces include diffusive deposition from a vapor or liquid phase absorbent (organosilanes) to hydrophilic substrates,[79–83] a density gradient method,[84] spatially varying electrochemical desorption/adsorption of alkanethiols on gold electrodes,[45] hyperthermal polyatomic ion deposition,[85] atom transfer radical polymerization (ATRP) (grafting from initiator gradients generated via diffusion,[86,87] continuous depletion of monomer solution[86,88]), thermochemical manipulation of aliphatic tert-butyl ester–functionalized self-assembled monolayers,[89] and continuous immersion techniques (polyvinyl carbonate films in NaOH solution,[44] gold substrate in alkanethiol solutions,[90] gradient chemisorption in ATRP initiator solution,[78] and a metal oxide substrate into a solution of polycationic polymer [electrostatic interaction]).[91] These methods result in a gradient in surface functional group density that can generally be translated into gradients in polymer grafting density (or molecular weight), surface nanoparticle density (by covalent or charge[92,93] interactions), and/or immobilized bioactive factor density.

Development of 2D gradient substrates with continuously modified polymer compositions has been one of the central themes in combinatorial polymer research.[94,95] Gradients in thickness and nanostructures were achieved by block copolymer thin film casting using accelerated knife-edge coating followed by annealing.[96,97] A gradient in morphology and nanoscale roughness was achieved by using a constant-thickness thin film casting followed by temperature gradient annealing.[98] Utilizing a three-syringe pump system, polymer composition gradients were generated by thin film casting followed by annealing, where an appropriate choice of polymers along with acceleration of the knife-edge coater and annealing temperature can lead to gradients in other properties (such as thickness, chemistry, crystallinity, stiffness, microstructure, roughness, wettability, degradation rate, or a combination).[99–103] In particular, coating velocity and acceleration affect the thickness of the film, and temperature affects the morphological appearance and roughness (as rate of crystallite nucleation is a function of temperature).[98] By combining UVO pretreatment, surface energy gradients were also introduced to the substrates.[104] Utilizing one or a combination of these continuous gradient-generation methodologies, 2D "orthogonal" gradient libraries were also created, where gradual variation in one or more properties occurred orthogonal to each other independently; for example, orthogonal gradients of molecular weights or molecular weight–graft density generated through ATRP, or an orthogonal gradient in the thickness (h), temperature-induced roughness (T) or composition (ϕ) of polymeric thin films (i.e., h-T, h-ϕ, and T-ϕ libraries).[88,101–103,105–107]

To summarize, a number of 2D surface physicochemical gradient-generation methodologies have been developed in the past and have served as a great high-throughput fast-screening tool. An important future step from a tissue engineering perspective will be the advancement of such methodologies from 2D flat substrates to 3D polymeric scaffolds, and recording/validating the phenomena observed on 2D substrates in a 3D environment, as cellular responses in 2D environments are known to be different from those in 3D environments.[108,109]

Metal or Bioceramic Materials

A major area of contemporary research is the development of biomaterial-based implants for orthopaedic and orthodontic applications (including maxillofacial, hip, or knee

Table 3. Methods to Create Surface Chemistry Gradients on 2D Substrates Including Functional Group Gradients and Oligomer/Polymer-Grafted Surfaces

		Materials[a]						
Method	*Possible gradient controlling parameter(s)*	*Grafted species*	*Substrate*	*Final-graded functionality/ surface characteristics*	*Gradient type(s) characterized*	*Bioactive or model factors used (if any)*	*Cell type(s) investigated (if any)*[b]	*References*
Diffusive deposition (vapor or liquid phase adsorbent)	Diffusion controlling factors	Dichlorodimethylsilane or other organosilanes (hydrophobic)	Hydrophilic substrates (silicon dioxide, silica, quartz)	Hydrophobic (methyl groups) gradient on hydrophilic substrate	Functional group density, wettability	Fibrinogen, γ-globulin, lysozyme, kininogen, IgG	–	79–83
Grafting from initiator gradients generated via diffusion (ATRP and/or NMRP)[c]	Time, monomer concentration, temperature[#]	PAA/(PMM & PS) grafted from organosilane-based initiators	Silicon wafer	(−COOH) functionality	Polymer graft density, thickness, wettability	–	–	86, 87
Continuous depletion of monomer solution (ATRP)	Monomer solution removal rate, MCl_2 concentration (governing reaction rate and polydispersity)[&]	PMM, (PHEMA and PMM)	Silicon wafer with chemisorbed initiator	(−COOH) functionality	Molecular weight (orthogonal), thickness	–	–	86, 88
Initiator gradient via diffusion–continuous depletion of monomer solution (ATRP)	(As mentioned above)[#,&]	PDMAEMA, PHEMA	Silicon wafer	(−COOH) functionality	Polymer graft density & molecular weight (orthogonal) (others, e.g., thickness, wettability)	Lysozyme, fibronectin	Osteoblast-like cells	88, 105
Thermochemical manipulation of aliphatic tert-butyl ester functionalized SAM	Temperature(s), pH, nanoparticle colloidal solution properties	($-NR_2$) and (−COOH) functionalized polystyrene nanospheres	Gold substrate with functionalized SAM	Nanosphere gradient (built on SAM containing −COOH gradient)	Functional group density, nanoparticle density	–	–	89
Density gradient method	Reactant concentration, reaction time (i.e., flow rates)	Dichlorodimethylsilane or other organosilanes (hydrophobic)	Hydrophilic substrates (silicon dioxide, silica, quartz)	Hydrophobic (methyl groups) gradient on hydrophilic substrate	Functional group density, wettability	Fibrinogen, IgG, lysozyme	–	84

Hyperthermal polyatomic ion deposition	Flurocarbon ($C_3F_5^+$) ion fluence (ions/cm^2)	Flurocarbon	PMM, PS	Fluorocarbon	Functional group density, wettability	–	–	85
Spatially varying electrochemical desorption/ adsorption of alkanethiols	Applied potentials, time	Carboxylic acid–modified PS nanospheres	Gold electrode with assembled amine-terminated alkanethiol layer	($-NH_2$) functionality translated into PS surface	Functional group density, nanosphere surface density	–	–	45
Continuous immersion in NaOH solution	Immersion speed (time), temperature, [OH]	(Hydrolysis of the surface groups)	PVC films	Carbonate (hydrophobic) and hydroxyl (hydrophilic) groups	Functional group density, wettability	–	Endothelial cells	44
Continuous immersion of gold substrate in alkanethiol solution	Immersion speed (time)	Methyl- and hydroxyl-terminated alkanethiol	Gold-coated silicon wafers	($-CH_3$) or ($-OH$) groups	Functional group density, wettability	–	–	90
Continuous immersion in initiator solution (ATRP)	Immersion speed (time)	PHEMA	Silicon wafer	($-COOH$) functionality	Functional group density	Fibronectin	Fibroblasts	78
UV (or photo-) irradiation	UV properties, exposure time using a motorized stage, exposure intensity using a mask or filter[α]	Etching (ozonolysis)	Silane monolayer (on glass), polymeric substrates (PCL)	Hydrophilic functionalities ($-OH$, $-COOH$)	Surface energy, wettability	Fibronectin	Osteoblast-like cells	49, 50
		Etching (ozonolysis)	Silane monolayer on silicon substrate masked with a elastomeric stamp	Hydrophilic ($-COOH$ groups) gradient on hydrophobic SAM (methyl groups)	Surface energy, wettability	–	–	51
		BP–TEG–PE polymerization (using a heterobifunctional photolinker)/ BP-RGD	Glass, alkanethiolate monolayer on gold-coated silicon wafer	Model factor	Graft density, model factor	R-phycoerythrin	–	65, 66
		Graded preirradiation to induce $-COOH$ functionality followed by PAAcid grafting	Polyethylene terephthalate	Model factor and $-COOH$ functionality	Graft density, model factor	Laminin (covalently attached)	PC-12 cells	46

(continued)

Table 3. (Continued)

Method	Possible gradient controlling parameter(s)	Materials[a]			Gradient type(s) characterized	Bioactive or model factors used (if any)	Cell type(s) investigated (if any)[b]	References
		Grafted species	Substrate	Final-graded functionality/ surface characteristics				
		EBPDMA, TEGDMA	Glass	Functionality of casted polymers	Methacrylate conversion, mechanical properties	–	–	41
		MMA	SBDC monolayer on silicon wafer	(–COOH) functionality	Graft density, thickness, model factor	RGD	Fibroblasts	67
Radio-frequency gas plasma discharge	Plasma composition, electrode-substrate gap width, power, time, exposure area using a graded or partially covering mask/ exposure time using a moving mask, diffusion[β]	Peroxide initiators (surface oxidation or etching)	Polyethylene (inert), PDMS, PS, PTFE	Oxygen-based functionalities (such as hydroxyl, ester, acid, ether, ketone, or aldehyde groups)	Functional group density, surface energy, wettability	Constituents in blood, albumin, IgG, fibrinogen	Platelets	52–55
		ppAAm and ppHEX (graft copolymerization)	Glass	Hydrophobic alkane groups to hydrophilic allylamine groups	Thickness, functional group density, wettability	–	Fibroblasts	77
		ppAAm/octa-1,7-diene and ppAA (graft copolymerization)	Glass	Amine/hydrocarbon and carboxyl functionalities	Thickness, functional group density, wettability	IgG	–	74–76
UV irradiation–plasma discharge	(As mentioned above)[α,β]	AA	Polymeric substrate	Hydrophilic (–COOH)	Functional group density, wettability	Serum proteins	Neurons	64
Power-graded corona discharge treatment	Substrate translation velocity, electrode-substrate gap width, power, time	Peroxide initiators (surface oxidation)	Polyethylene (insert)	Oxygen-based functionalities (such as hydroxyl, ester, acid, ether, ketone, or aldehyde groups)	Functional group density, wettability	Fetal bovine serum, calf serum and NGF, human albumin, plasma proteins	CHO cells, fibroblasts, endothelial cells, PC-12 cells, platelets	56–61
		″	PLGA/ polycarbonate	″	″	Fetal bovine serum	Fibroblasts	62, 63

		PEO-MA	Polyethylene (inert)	PEO	Functional group density, wettability	Plasma proteins	Platelets	68, 69
		AA/NaSS/ DMAPAA (graft copolymerization), MAPC (graft copolymerization)	Polyethylene (insert)	Charged functionalities (AA/NaSS: −ve, DMAPAA: +ve)	Functional group density, charge density, wettability	Plasma proteins	CHO cells, platelets	70, 71
		MAPC (graft copolymerization)	Polyethylene (insert)	Groups with high phospholipid affinity	Functional group density, wettability	Plasma proteins, fibronectin	Platelets, fibroblasts	72, 73
Thin film casting (using knife-edge flow coating) followed by annealing	Polymer solution composition, annealing temperature(s) and time, knife-substrate gap width, coating velocity and acceleration	PS-b-PMM	Silicon wafer	PS (function of film thickness)	Thickness, nanostructure	–	–	96, 97
Thin film casting followed by annealing (utilizing a temperature gradient)	Annealing temperature(s) and time[δ]	PLLA	Silanized silicon wafer	Functionality of casted polymers	Crystallinity, nanoscale roughness	Fetal bovine serum	Osteoblast-like cells	98
	(As mentioned above)[δ]	PS	Silicon wafer	PS	Thickness and temperature (orthogonal) (others, e.g., roughness)	–	–	106
Gradient mixing (with a three-syringe pump system)–thin film casting–melt annealing	Sample collection rate, annealing temperature(s) and time, knife-substrate gap width, coating velocity[η]	PDLLA & PLLA	Glass	Functionality of casted polymers	Material composition, crystallinity, stiffness, roughness	–	Osteoblast-like cells	99, 100
Gradient mixing–melt annealing (utilizing a temperature gradient)	(As mentioned above)[δ,η]	(PDLLA & PCL)/(PS & PVME)/ (PLGA & PCL)	Glass, silicon wafer	″	Material composition and temperature (orthogonal) (others, e.g., chemistry, microstructure, crystallinity, roughness, hydrophilicity, stiffness, degradation rate)	–	Osteoblast-like cells, VSMCs	101–103

(continued)

Table 3. (Continued)

Method	Possible gradient controlling parameter(s)	Materials[a]			Gradient type(s) characterized	Bioactive or model factors used (if any)	Cell type(s) investigated (if any)[b]	References
		Grafted species	Substrate	Final-graded functionality/ surface characteristics				
UV (ozonolysis) treatment–thin film casting–annealing	(As mentioned above)[α,δ]	PS-b-PMM with an underlying oxidized chlorosilane monolayer	Silicon wafer	(PS or PMM) (function of surface energy)	Surface energy and thickness (orthogonal) (others, e.g., microstructure)	–	–	104
Electrostatic interaction (continuous immersion of a charged substrate into a suspension of nanoparticles carrying the opposite charge)	Immersion rate/colloidal solution filling rate	Anionic nanospheres (silica, gold, silver)	Poly(ethylene imine)-coated silicon wafer, glass slide modified with cationic moieties	PLL-g-PEG-RGD–coated nanospheres/ protein-conjugated nanospheres	Nanoparticle density gradient, nanostructure, nanoscale roughness, protein gradient	Bovine serum albumin, ephrin-A5, ephrin B1	Osteoblasts, hippocampal cells	92, 93
Electrostatic interaction (continuous immersion of a metal oxide substrate into a solution of polycationic polymer)	Immersion rate	PLL-g-PEG	TiO_2/Nb_2O_5	Functionality of grafted polymer, surface-adsorbed protein	Polymer graft density, thickness, adsorbed or conjugated protein surface density	Human serum albumin, fibrinogen, IgG, blood serum and blood plasma	–	91

[a]PAA: poly(acrylamide); PMM: poly(methyl methacrylate); PS: polystyrene; PHEMA: poly(2-hydroxy ethyl methacrylate); PDMAEMA: poly(dimethyl aminoethyl methacrylate); SAM: self-assembled monolayer; PVC: poly(vinyl carbonate); PCL: poly(ε-caprolactone); BP: benzophenone; TEG: tetraethylene glycol; PE: polyethylene; RGD: (arginine-glycine-aspartic acid); PAAcid: poly(acrylic acid); EBPDMA: ethoxylated bis-dimethacrylate; TEGDMA: triethylene glycol dimethacrylate; MMA: methacrylic acid; SBDC: N,N-(diethyl-aminodithicarbamoylbenzyl(tri-methoxy)silane); PTFE: poly(tetrafluoro ethylene); ppAAm: plasma polymerized allylamine; ppHex: plasma polymerized hexane; ppAA: plasma polymerized acrylic acid; PLGA: poly(lactic-co-glycolic acid); PEO-MA: polyethylene oxide-monomethacrylate; NaSS: sodium p-styrene sulfonate; DMAPAA: N,N-dimethyl aminopropyl acrylamide; MAPC: ω-methacryloyloxyalkyl phosphorylcholine; PS-b-PMM: polystyrene-b-poly(methyl methacrylate); PLLA: poly(L-lactic acid); PDLLA: poly(D,L-lactic acid); PVME: poly(vinyl methyl ether); PLL-g-PEG: poly(L-lysine)-graft-poly(ethylene glycol); PDMS: polydimethylsiloxane.

[b]PC-12: pheochromocytoma; CHO: Chinese hamster ovary; VSMCs: vascular smooth muscle cells.

[c]ATRP: atom transfer radical polymerization; NMRP: nitroxide-mediated radical polymerization.

NGF: nerve growth factor.

replacements). Unlike tissue-engineered constructs, such implants are usually nondegradable and devoid of cells by design, usually lacking in self-repairing ability. Nevertheless, such "spare-parts" appear promising due to their "off-the-shelf" nature. Two major categories of such implants are metallic implants and bioceramic implants. Gradient-based strategies can be integrated in the design of such implants to address some of the major concerns associated with these implants. In this section, we highlight some of the interesting and relevant current trends in implant design (Table 4).

Due to the relatively inferior biocompatibility and poor corrosion resistance of metallic implants, bioceramic or polymeric coatings are usually applied. The bioconductive nature of bioceramic coatings, or degradable nature of the polymeric coatings, imparts on them the ability to induce/promote bone ingrowth and to help in the integration of the implant with the surrounding tissue. However, the durability of the coating–substrate interface is a common concern.[110] This issue has been addressed by using coatings made of so-called functionally graded materials, which are nonuniform composites containing a continuous or multilayered structure, varying in composition (and other desired properties) from one end of the composite to the other.[110–113] Using a bioconductive ceramic (such as hydroxyapatite, β-tricalcium phosphate, or bioglass) as the outer surface, a metallic/tough bioceramic (TiO_2, Al_2O_3, ZrO_2, etc.) as the inner surface (contacting the substrate) and a gradient of the materials in between, the desired balance between the mechanical properties and bioconductivity can be achieved.[110,114,115] In a similar manner, graded polymeric coatings or graded composite (polymer–bioceramic) coatings were employed to achieve gradient transitions in mechanical properties and degradation, where degradation of the coating can be programmed to match the bone ingrowth, ideally.[116] Polymeric coatings can also be utilized as a controlled delivery vehicle of osteogenic factors that may enhance bone ingrowth. Likewise, gradient surface treatments of metal implants were utilized to reduce the metal ion release, enhance corrosion resistance, and improve the biocompatibility of the surface layer, while preserving the superior mechanical properties of the implant with a durable interface between the coating and the implant.[117–119] For example, electrochemical oxidation processes were used to form graded TiO_2 coatings on Ti-based implants with a porous outer coating to enhance integration with the bone, and a dense inner coating to reduce metal ion release.[119] Similarly, addressing wear-induced osteolysis as a result of ultra-high-molecular-weight polyethylene (UHMWPE) debris formation following total joint arthroplasty, a gradient surface treatment using a low-energy electron beam resulted in depth-dependent gradient crosslinking that yielded a UHMWPE surface with high wear resistance and superior mechanical properties in the interior (due to low crosslinking).[120,121] In a different approach, a gradient interpenetrating polymer network, formed due to the diffusion of poly-L-lysine (PLL) into UHMWPE, was proposed to address UHMWPE wear, where recruitment of hyaluronic acid by PLL (via charge interaction) was hypothesized to decrease joint friction and wear.[122]

Another area of application for gradient-based strategies is in the design of the implant itself. To address detrimental bone resorption resulting from stress shielding, a metal implant with a gradient in porosity was utilized to match the stiffness of the implant to that of the bone.[123] Graded structures were created to mimic the bone architecture (low porosity outside, as in cortical bone, and high porosity inside, as in cancellous bone)[112,124] or to influence the bone ingrowth while maintaining the mechanical integrity of the implant (high porosity inside and low porosity outside).[125,126] These two approaches to create bimodal bone structures primarily depend on the desired mechanical characteristics of the engineered structure and influence the pore sizes. Functionally graded implants have also been considered for other applications, for example, for the treatment of cranial defects[127] and spinal disc prosthesis.[128] Moreover, gradient-based structures may be instrumental in optimizing the biocompatibility of the implants. Such structures can be utilized as cost-effective fast-screening tools in determining the biocompatibility of the materials, as demonstrated earlier,[129] or can be utilized in the design of implants, both traditional and functionally graded implants, to improve biocompatibility at desired locations.[130,131]

Various techniques have been applied to create functionally graded implants and coatings, resulting in desired spatial variation in properties of interest, while reducing or eliminating the interfacial stresses due to the material-property disparity that may cause delamination[1,110] (summarized in Table 4). Many studies, at least preparation-wise, included the formation of graded structures in a multi-stepwise, as opposed to continuous, manner. Although these techniques may have resulted in the formation of micro/submicron-range diffusion-based gradients at the interface, it may not be sufficient to avoid delamination or interfacial failure. Controlled continuous gradation can be employed to ensure a smooth transition, as opposed to stepwise transition, that may (or may not) be a concern for delamination. Another distinct benefit with the use of continuously graded structures is that many techniques for creating such continuously graded structures offer a one-step fabrication method, which is more efficient than multiple processing steps commonly employed for stepwise gradation.[132] For example, a dual-torch plasma spraying was used to create decreasing titanium and increasing hydroxyapatite gradients toward the surface by independently adjusting the feed rate and plasma power of mixture gases to yield a continuously varying gradient region.[110] Generally, a higher percent value of the thickness of the gradient region compared to the overall thickness of the implant/coating can be selected to eliminate the cause for concern (i.e., delamination); however, one must also take the envisioned application into consideration.

In summary, functionally graded materials have tremendous application in the design of implants/implant coatings, which can either be fabricated or may result as an effect of transport-based surface-treatment methods (see reviews by Mortensen and Suresh[133] and Kieback *et al.*[134]). Compared to multi-stepwise graded structures, continuous gradient–based approaches appear promising as they may provide more time-efficient and mechanically robust alternatives; however, they are still in their infancy. In this regard, development of novel fabrication techniques and comparison with the corresponding stepwise gradation will provide more insight regarding the usefulness of continuously graded structures.

"Chemical Signal" Gradients

Concentration gradients of bioactive signaling molecules (hereafter, collectively referred to as chemical signals) play a

Table 4. Gradient-Based Approaches Highlighting Current Trends of Application in Implant Design

Application	*Gradient type (transition type)*	*Fabrication method*	*Geometry/gradient direction*	*Implant/coating thickness scale*	*Thickness scale of the gradient region or number of step transitions*[a]	*Materials used*[b]	*References*
Bioceramic materials with bimodal pore structure for bone replacement	Porosity and pore size (step gradients)	Differential impregnation–heat sintering	Cylindrical/axial	≥mm	One transition	HA and a cellulosic sponge	124
		Dip casting–vacuum impregnation–stitching/press fitting	Cylindrical/radial	≥mm	One transition	HA/TCP and polymeric foams	112
		Multiple slip-casting–heat sintering	Cylindrical/radial	≥mm	One transition	HA, PVC, and Li_3PO_4	125
		Multiple tape-casting–sintering–heat-induced removal of porogens	Bar or disc/axial	≥mm	Four transitions	HA, PBMA	126
Graded orthopaedic replacement to reduce stress-shielding	Porosity and stiffness (step gradients)	Liquid phase sintering	Bar/longitudinal	≥mm	Two transitions	Ti, Si powder	123
UHMWPE wear resistance (for total joint arthroplasty, etc.)	Crosslinking density (continuous gradients)	Irradiation (low-energy electron beam)	Finished acetabular liner/radial	≥mm	mm	UHMWPE	121
		Melt-irradiation (low-energy electron beam)	Hemispherical/radial	≥mm	mm	UHMWPE	120
	Material composition [diffusion-based IPN[c]] (continuous gradient)	Swelling at elevated temperature with ultrasonics	–	>monolayer	–	UHMWPE, PLL	122

Bioceramic coatings on metallic implants	Material composition (step gradients)	Plasma spray	–	660 μm (coating thickness)	Three transitions	HA, Ti-6Al-4V, TCP/TiO_2	114
		Plasma spray	–	–	Three transitions	HA, Ti	115
	Material composition (continuous gradients)	Plasma spray	–	100 μm	40 μm	HA, Ti	110
Bioceramic polymer–based skull implant (e.g., calvarial defect repair)	Material composition and porosity (step gradients)	Hot pressing–gas foaming	Custom (CAD/CAM assisted)	~cm	Four transitions	PLA, $CaCO_3$, $Ca_3(PO_4)_2$	127
Bioceramic polymer intervertebral disc prosthesis	Material composition (continuous gradient)	Centrifugation	Cylindrical/radial	≥mm	Across the sample (100%)	HA, polycarbonate-urethane	128
Polymeric coatings on metallic implants	Material composition (step gradients)	Dip coating	–	1 mm (coating thickness)	Two transitions	Ti, PLA, HA, $CaCO_3$	116
Gradient surface treatment of metallic implant to improve biocompatibility/ osteoconductivity	Material composition (sodium titanate and apatite)	Sodium hydroxide treatment–heat treatment	–	≥mm	Nanoscale	Ti-6Al-4V, sodium titanate	117
	(Continuous gradient) material composition (apatite) (step gradient)	Sol-gel and slurry dip coating–drying and sintering	–	<100 μm	Three transitions	ZrO_2, HA, fluorapatite	118
	Degree of oxidation (porous outer layer–dense inner layer) (continuous gradient)	Electrochemical oxidation (preanodic oxidation–microarc oxidation)	–	16 μm (coating thickness)	<1 μm (nanoscale)	Ti, TiO_2	119

(continued)

Table 4. (Continued)

Application	*Gradient type (transition type)*	*Fabrication method*	*Geometry/gradient direction*	*Implant/coating thickness scale*	*Thickness scale of the gradient region or number of step transitions*[a]	*Materials used*[b]	*References*
As a fast-screening tool for biocompatibility assessment of the metallic implants	Material composition (step and continuous gradient)	Sedimentation/powder packing–sintering (furnace/high-frequency induction heating/spark plasma sintering)	Cylindrical/axial	≥mm	(See the articles for details)	Ti, HA (and others)	130, 131
	Material composition (step gradients)	Powder packing–hot isotactic pressing	Bar	≥cm	Nine transitions	Ti, Ni	129

[a]For continuous gradient approaches, % thickness of the gradient region compared to the overall thickness of the structure is indicated. To eliminate/reduce interfacial stresses that may cause delamination, a higher % value of the thickness of the gradient region compared to the overall thickness of the implant/coating can be selected. However, one must also take the envisioned application into consideration. Some of the studies utilized step gradients, where dual/multiple layers were present having sharp (discontinuous) interfaces in between. The diffusive effects may have led to a blurred region at the interface; however, we do not treat it as a continuous gradient unless measured. Studies, where multilayered structures were created and the overall gradient region comprised of several step transitions that spanned the entire implant/coating, are marked by the number of such transitions.

[b]HA: hydroxyapatite; TCP: tricalcium phosphate; PVC: polyvinyl polyacrylate; PBMA: poly(butylmethacrylate); UHMWPE: ultra-high-molecular-weight polyethylene; PLA: poly(lactic acid).

[c]IPN: interpenetrating network.

crucial role in developmental and biological repair processes, including morphogenesis, wound healing, the immune response, vessel pathfinding, and axonal guidance, where cellular migration and/or differentiation is sensitively governed by spatially patterned endogenous chemical signals (see reviews by Gurdon and Bourillot,[135] Eichmann *et al*.,[136] Tessier-Lavigne,[137] and Parent and Devreotes[138]). A biomimetic approach toward tissue regeneration necessitates a proper consideration of the spatial and temporal aspects of exogenous delivery of such signals in tissue engineering.[139,140] The following sections contain a brief discussion of the importance of chemical signal gradients, and the techniques applied to generate chemical signal gradients in 2D and 3D environments.

Gradients of Growth/Differentiation Factors and Cell Adhesion Molecules

Spatial patterning of chemical signals is a field of growing interest for the tissue engineering community. Several of these bioactive factors are well characterized for different tissue engineering applications, are known to induce concentration-dependent cell type–specific responses, and usually work in a synchronized manner with other similar factors during the development or repair of a natural tissue.[141] While these factors are traditionally delivered homogeneously for *in vitro* or *in vivo* tissue engineering, both temporal and spatial control over the delivery of such factors is an understood requirement for biomimetic repair and regeneration.

Cell–ECM interfacing is governed through a type of ligand-receptor binding primarily mediated by transmembrane adhesion proteins of the integrin family, where extracellular domains of the integrin receptors form anchoring junctions (such as focal adhesions, fibrillar adhesions, and hemidesmosomes) by binding to certain ECM proteins, for example, collagens, fibronectin, fibrinogen, or vitronectin.[142] Cell–matrix interaction, in turn, produces specific cell responses that influence cell adhesion, motility, shape, orientation, differentiation, and survival. Specific parts of the ECM protein sequences act as cell adhesion ligands, and domains, RGD, YIGSR, and IKVAV represent some well-investigated peptide sequences that are recognized by cells as adhesion sites (see reviews by Yamada[143] and Hersel *et al*.[144]). The RGD sequence, for example, is a cell adhesion site found in active fibronectin, fibrinogen, and laminin.

Continuous gradients of chemical signals are a form of spatially patterned signals that have been successfully developed and employed in various investigations, most notably, probing directed axonal regeneration,[46,145–152] nerve regeneration,[153] controlled cellular migration, and localization and/or alignment involving fibroblasts, endothelial cells, Chinese hamster ovary cells, vascular smooth muscle cells, leukocytes, and neutrophils.[42,67,73,78,154–163] In their soluble or immobilized forms, the chemical signal gradients induce specific cellular responses, which may include controlled cellular migration (a.k.a. chemotaxis or haptotaxis, respectively) (Table 1), usually in the direction of increasing concentration/surface density of the chemical signal. A positive effect on directed axonal growth has been demonstrated under the influence of various chemical signal gradients, including gradients of IKVAV-containing peptide,[164] laminin,[145–147] nerve growth factor (NGF),[148,150] combined laminin and NGF,[153] and combined NGF and neurotrophin-3,[149,151] where neurite extensions were found to be superior in the presence of signal gradients compared to corresponding homogeneously delivered signals. Wound healing is another area of investigation. Controlled movement of fibroblasts is known to take place under the influence of chemotactic factors secreted by macrophages and platelets[165] and represents a key area to explore the effect of various chemical signal gradients on the migratory behavior of fibroblasts, leukocytes, and neutrophils. In addition, the ability of chemical signal gradients (such as an RGD-containing peptide density gradient) to influence the alignment of the fibroblasts, as suggested by the authors, can also be exploited in the tissue engineering of ligaments and tendons.[154] Moreover, gradient substrates can also be used as a screening tool in optimizing the dosage of growth factors that lead to, for example, a higher cell proliferation rate or improved juxtracrine signaling.[158] In addition to chemical signals, a number of other model factor gradients (such as other proteins or fluorophores) have also been created during the development of gradient-generation techniques, some of which are summarized in Table 5.

Strategies to Create Gradients of Growth/Differentiation Factors and Cell Adhesion Molecules

Chemical signal gradients can be broadly divided into two categories: soluble and immobilized. Other important categories include gradients in two dimensions versus three dimensions, and the principle involved in gradient generation. Specifically, the methods to create signal gradients are based on either diffusion- or convection-based approaches. In convection-based approaches, a gradient of the concentration/surface density of the bioactive factor is achieved by a gradual increase/decrease in the concentration of the factor itself, or continuous spatial variation in the chemistry/preprocessing of the substrate that leads to a gradient in the concentration/surface density of the factor. General methods used to create chemical signal gradients (for peptide and proteins) are summarized in Table 6.

Soluble

To study chemotactic responses of cells under the influence of gradients, the principle of molecular diffusion was first utilized to create soluble factor gradients in solutions (such as culture medium) using a Boyden chamber or its variants (such as Zigmond and Dunn chambers).[159,161,162] These chambers, although simple and inexpensive, have drawbacks in sustaining the concentration gradients of signals for a long period and do not provide a 3D cell culture environment.[166] Similar creation of gradients in macroporous gels (such as agarose, fibrin, or collagen) provided 3D culture platforms. Approaches to generate signal gradients in such gels involve either a single source/chamber of bioactive factors (such as a chamber of bioactive factor–rich solution),[157,167] or multi-source/chamber of factors (such as the gel in between two chambers of factor, or delivery of factors at multiple positions in gel)[150,151,160,163]; the latter enabling the generation of relatively more stable linear gradient profiles. A controlled microdispensing technique was recently developed to create patterns of chemical factors with user-defined profiles on the surface of thin 3D gels, where gradients of these factors are established via diffusion in the gel, which were found to be

Table 5. Example Studies Involving Concentration/Surface Density Gradients of Peptides, Proteins, or Other Model Factors for Tissue Engineering

Factors[a]	*2D/3D*	*Gradient type*		*Materials used to incorporate the factor*[b]	*Notes*	*Application*	*Cell type investigated*[c]	*References*
		Profile	*Soluble/ immobilized*					
CAMs/peptides								
RGD-containing peptides	2D[d]	Linear	Immobilized (covalently bound)	Poly(acrylic acid), poly(methacrylic acid) (photoiniferter SAM), poly(ethylene glycol)	Acryloyl derivatization to induce photopolymerization	Controlled cell adhesion, alignment, and motility (temporally and spatially)	Fibroblasts, endothelial cells	42, 67, 154, 156
	3D	Nonlinear	Soluble	Collagen or fibrin gel	–	Studying chemotaxis	Fibroblasts	157
IKVAV	2D	Linear	Immobilized (covalently bound)	Polystyrene substrate	Benzophenone derivatization to induce photopolymerization	Probing axon guidance	DRG neurons	164
Laminin	2D	Linear	Immobilized (adsorbed or covalently bound)	Glass/poly-L-lysine–coated substrate, carboxy-terminated alkanethiol monolayer on gold	–	Probing neuronal development and axon guidance, cell migration	Hippocampal neurons, intestinal IEC-6 cells	146, 147, 170
	2D	Sigmoidal	Immobilized	Polyethylene terephthalate	Carbodiimide chemistry	Probing axon guidance	PC-12 cells	46
	3D	Quadratic	Immobilized (covalently bound)	Agarose	SANPAH (heterobifunctional crosslinker), photoreactive perfluoro arylazide group	Improved neurite outgrowth and nerve regeneration in 3D environment	DRG neurons, sciatic nerve regeneration (*in vivo*)	145, 153
Fibronectin	2D	(Function of alkyl chain length)/ sigmoidal	Immobilized (adsorbed)	ω-Methacryloyloxyalkyl phosphorylcholine–grafted PE/poly (2-hydroxyethyl methacrylate)	–	Manipulating cell adhesion and spreading	Fibroblasts	73, 78
Growth factors								
EGF	2D	Custom	Immobilized (covalently bound)	Azidophenyl-derivatized poly (allylamine)-coated polystyrene	Azidophenyl derivatization	Concentration-dependent effects of immobilized biosignals on cellular mobility and localization, artificial juxtracrine simulation	Chinese hamster ovary cells	158

bFGF	2D[c]	Linear	Immobilized (covalently bound)	Poly(ethylene glycol)	Acryloyl derivatization to induce photopolymerization	Directed cellular migration and alignment	VSMCs	155
NGF	3D	Custom/linear	Soluble	Collagen gel/ agarose gel	–	Axonal guidance, studying chemotaxis and morphogenesis	DRG neurons/PC-12 cells	148, 150
		Linear	Immobilized (entrapped)	Poly(2-hydroxyethyl methacrylate)	–	Axonal guidance	PC-12 cells	152
NGF and NT-3	3D	Linear	Soluble	Agarose	–	Axonal guidance	DRG neurons	151
	3D	Linear	Immobilized (entrapped)	Poly(2-hydroxyethyl methacrylate)	–	Axonal guidance	DRG neurons	149
Model proteins and others								
Human serum	2D	Nonlinear/ linear/linear	Soluble	Culture medium	–	Studying chemotaxis	Polymorphouclear leukocytes/ neutrophils	159, 161, 162
cAMP	3D	Linear	Soluble	Agarose gel	–	Studying chemotaxis	*Dictyostelium discoideum* amoebae	163
ZAS	3D	Linear	Soluble	Agarose gel	–	Studying chemotaxis	Leukocytes	160
Casein-b	3D	Custom	Soluble	Collagen gel	–	Axonal guidance, studying chemotaxis and morphogenesis	–	166
HRP	3D	Linear	Immobilized	Silk fibroin	Carbodiimide chemistry	Chemotaxis, tissue engineering, biosensors	–	174
R-phycoerythrin	2D	Linear	Immobilized	Polystyrene substrate	Carbodiimide chemistry, benzophenone derivatization	Probing biological responses	–	66
Fluorescent dye, antigens and/or antibodies	2D	Custom	Soluble/ surface adsorbed	PDMS	–	Chemotaxis, haptotaxis, etching, nucleation and growth, Marangoni effects, antibody-antigen binding	–	168, 175

[a]RGD: arginine-glycine-aspartic acid; SANPAH: sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; NGF: nerve growth factor; NT-3: neurotrophin-3; ZAS: zymosan-activated serum; HRP: horseradish peroxidase; CAMs: cell adhesion molecules; cAMP: 3′-5′-cyclic adenosine monophosphate.

[b]PDMS: polydimethylsiloxane; SAM: self-assembled monolayer; PE: polyethylene.

[c]DRG: dorsal root ganglion; PC-12: pheochromocytoma; VSMCs: vascular smooth muscle cells.

[d]Some of these studies (e.g., Delong *et al.*[156]) employed the techniques that were capable of generating gradients of immobilized factors in 3D. However, cellular interactions were monitored on the surfaces of the gels (i.e., in 2D).

Table 6. Methods to Create Surface Density/Concentration Gradients of Peptides and Proteins for Tissue Engineering Applications

Fabrication							
Technique	*Process driving phenomenon(-a)*	*Additional processing*	*Primary gradient-shape controlling parameter(s)*	*Gradient scale*	*2D/3D*	*Gradient-shape control?*	*References*
Soluble factor gradients							
Boyden/Zigmond/Dunn chambers	Diffusion (in solution)	–	Time	Macroscale	2D[a]	Limited	159, 161, 162
Single-source/chamber (of bioactive factor) approaches	Diffusion (in macroporous gels)	–	Time	Macroscale	3D	Limited	157, 167
Dual-/multisource/chamber approaches	Diffusion (in macroporous gel)	–	Time	Macroscale	3D	Limited	150, 151, 160, 163
	Monomer flow, diffusion (in solution)	Microfluidics[b]	Channel design, monomer flow	Micro- to macroscale	2D	Yes	168, 169, 171
Microprinting	Convection and diffusion (in macroporous gels)	Contact-less controlled microdispensing	Droplet ejection rate, stage translation rate	Macroscale	2D or 3D	Yes	148, 166
Soluble factor gradients (controlled release approach)[b]							
Phosphatidyl choline–based lipid microtubules (LMTs) in gels	LMT loading in gels	Gelation by cooling	LMT loading profile (the step size)	Macroscale	3D	Limited	153
Microsphere-based scaffolds	Matrix preparation using microspheres loaded with the active factors	Melding the microspheres (using heat-sintering, ethanol-melding, or dichloromethane treatment)	Microsphere size, polymer properties (degradation, molecular weight, etc.)	Macroscale	3D	Limited	172, 173
Immobilized factor gradients							
Single-source/chamber approaches	Diffusion (in macroporous scaffolds) (covalently bound)	EDC-NHS chemistry	–	Macroscale	3D	Limited	174

	Diffusion (adsorption on surface)	Capillary method	–	Macroscale	2D	Limited	146
Dual-source/ chamber approaches	Diffusion (in gels)	Heterobifunctional crosslinker	–	Micro- to macroscale	3D	Limited	145, 153
Pump-/gravity-driven flow of factor solution	Convection (in macroporous scaffolds)	EDC-NHS chemistry	Suction rate	Macroscale	3D	Yes	174
	Convection (monomer flow)	Photopolymerization (factor entrapped)	Monomer flow rate	Micro- to macroscale	2D or 3D	Yes	149, 152
		Photopolymerization (factor covalently attached)	Monomer flow rate	Micro- to macroscale	2D or 3D	Yes	154–156
	Monomer flow, diffusion	Microfluidics, photopolymerization (factor covalently attached)/ factor adsorbed	Channel design, monomer flow	Micro- to macroscale	2D	Yes	42, 147, 170
Capillary force–driven flow of factor solution	Monomer flow, diffusion (adsorption on surface)	Microfluidics (factor adsorbed)	Capillary forces, channel geometry, substrate affinity for the factor	Micro- to macroscale	2D	Limited	175
Grafting/ micropatterning	Differential photoexposure (photomask)	Protein conjugation to a photoreactive species	Mask pattern	Micro- to macroscale	2D	Yes	158
	Controlled-time exposure	Gradients of protein conjugated to a photoreactive species	Photoexposure time controlled using a motorized stage	Micro- to macroscale	2D	Yes	66, 164
		Polymer graft density/ thickness gradient (using motorized stage/ photomask or differential dipping in an initiator solution [ATRP])	Photoexposure time, mask pattern and/or initiator exposure time (filling/removal rate)	Micro- to macroscale	2D	Yes	46, 67, 78, 177
	Computerized printing (inkjet printing)	–	Pattern design, substrate and solution properties	Macroscale	2D	Yes	176

[a]Solution-based gradients can be generated in three dimensions. However, cells cannot be kept suspended and eventually attach to the substratum.
[b]Some approaches of gradient generation have potential to provide spatial as well as temporal control, such as, microfluidic-based and controlled release approaches.
EDC: ethyl(dimethylaminopropyl) carbodiimide; NHS: N-hydroxysuccinimide; ATRP: atom transfer radical polymerization.

stable for a day or more.[148,166] Laminar flow–based microfluidic devices have also been developed that are capable of generating concentration gradients of chemical signals with highly stable spatial and temporal profiles, although these have mostly been applied to 2D systems.[168–171] Devices based on controlled release principles, for example, phosphatidyl choline–based lipid microtubules (LMTs) loaded in gels or microsphere-based scaffolds, have recently been applied to create gradients of chemical signals/model factors.[153,172,173] Such devices may serve as long-term (several days to months) release vehicles for the generation of gradients of chemotactic factors.

Immobilized

The general techniques to create surface-immobilized chemical gradients include adsorption of the molecule on the desired surface, covalent linking of the peptides/proteins via peptide bond formation through carboxylic acid ($-COOH$) or primary amine ($-NH_2$) moieties present on the original or modified surface (for example, using carbodiimide chemistry), or by derivatizing with photoreactive moieties (such as azidophenyl, benzophenone, acryloyl, or aryl azide groups), all more or less governed by the chemistry of the substrate/scaffold, as reviewed earlier. General methods that have been utilized to create immobilized chemical signal gradients in two dimensions and three dimensions include single- or dual-source/chamber approaches,[145,146,153,174] pump- or gravity-driven flow of factor solution,[42,147,149,152,154–156,174] capillary-driven flow of factor solution,[175] automated printing,[176] and adsorption or covalent linking of proteins utilizing polymer-grafted/micropatterned substrates.[46,66,67,78,158,177] Microfluidics, photopolymerization, ATRP, and/or protein conjugation chemistry are among valuable flexible tools that were usually involved during the fabrication of immobilized protein gradients (Table 6).

Techniques to create bioactive factor gradients vary in terms of scale, accuracy, flexibility, and stability of gradient profiles.[166] In summary, hydrogel-based approaches have provided many routes to generate soluble or immobilized bioactive factor gradients in both two dimensions and three dimensions, which were widely used in studying the chemotaxis and haptotaxis. Surface modification and photopolymerization techniques have been successfully used to create gradients of surface-bound factors on polymeric surfaces. In contrast, fewer attempts have been made regarding the generation of such gradients in degradable 3D macroporous scaffolds, a prototype that constitutes a significant percentage of commonly used tissue engineering scaffolds. In this regard, primary issues to be addressed appear to pertain to the translation of many 2D physicochemical gradient-generation techniques from two dimensions to three dimensions.

Discussion

Integration of three schools of thought—functional, interfacial, and biomimetic tissue engineering—can be addressed through gradient-based strategies by combining physical and chemical signal delivery. Various tissues display strong nonhomogeneous characteristics in their morphology, cellular, and ECM organization. For example, cartilage (superficial, middle, and deep zones), bone (cortical and cancellous), blood vessels (media, intima, and adventitia), skin (dermis and epidermis), or any interfacial tissue (such as bone-cartilage or muscle-tendon), all consist of graded zonal structures to satisfy diverse functional needs. Gradients in mechanical properties often exist within and between the tissues, which help in avoiding stress concentrations,[2] for example, cartilage,[178] human crystalline lens,[179] and the dentin–enamel junction.[180] A tissue-engineered replacement must satisfy at least the "minimum" functional requirements that may be addressed through the choice of biomaterials and the scaffold design. Incorporating gradient-based physical signal delivery strategies in the design of biomaterials, such as pore-size and porosity gradients or stiffness gradients, may thus improve the functional characteristics of and cellular remodeling in the scaffolds. Simultaneously, chemical signal gradients that are involved during the regeneration and repair of tissues can be incorporated in the design of scaffolds. Gradients of chemical signals may also offer single cell source tissue regeneration alternatives for the regeneration of interfacial tissues, where a stem cell population can be selectively differentiated into disparate lineages in a graded manner in the same construct. Thus, a biomimetic approach can be combined with a functional tissue regeneration approach by utilizing a combined physical and chemical signal delivery through gradient-based strategies. As an example, stiffness gradients could be combined with growth factor gradients that may yield a synergistic response of enhanced axonal branching[36] and guided axonal regeneration.

While a combination of physical and chemical signal gradients may not be necessary for all tissues, such an approach could be an interesting subject of investigation for interfacial tissue regeneration. From the perspective of interfacial tissue regeneration, a transition from homogeneous cell/growth factor/scaffold designs to signal gradient-based tissue engineering may be advocated for a number of reasons. First, the replacement of a tissue that is engineered in isolation requires a fixation or bridging with the adjacent tissue (such as suturing, press-fitting, or gluing), which may not result in the best mechanical characteristics at the interface. Second, isolated tissue engineering may not be able to provide mutually inductive endogenous signals from the adjacent tissues that are involved during the tissue formation *in vivo*. In an extreme example, it is widely known that gastrulation and the subsequent fate of germ layers during embryogenesis depend on a series of spatially and temporally controlled inductive cell interactions.[181,182] In a specific example, relevant to osteochondral tissue engineering, an *in vitro* culture study reported that only coculture with chondrocytes (as opposed to fibroblasts or osteoblasts) was successful at promoting osteogenic differentiation of mesenchymal stem cells in a selective manner.[183] Even tumor cells display a strong neighbor-dependent behavior arising from cell-to-cell interactions.[184] Finally, stratified tissue regeneration techniques (e.g., utilizing bi- or multiphasic scaffolds), the closest alternative to gradient-based signal delivery, may not effectively mimic the native tissue function, or may undergo delamination due to stress concentrations.

Along with spatial regionalization of chemical signals, another key aspect of biomimetic tissue engineering is the delivery of such signals in a temporally controlled manner, that is, simultaneous or sequential release of multiple growth fac-

tors.[185,186] In this regard, controlled drug delivery technologies can be combined with gradient fabrication strategies, for example, phosphatidyl choline–based LMTs or microsphere-based scaffolds.[153,172,173]

In summary, continuous gradients of physical and chemical signals can be considered as an important subset of spatially patterned signals, capable of driving dynamic cellular phenomena and a cost-effective tool for high-throughput screening. A variety of gradient-generation techniques have been reviewed here, which are promising for biological and tissue engineering investigations. Controlled patterning of chemical signaling molecules combined with physical gradients of signals hold immense potential for complex tissue regeneration, which may be a missing ingredient in the quest to fulfill the true potential of the field of regenerative medicine. In the future, *in vivo* comparisons will be required to provide substantial evidence for the superior performance of gradient-based signal delivery strategies compared to traditional forms of tissue engineering.

Acknowledgments

The present work was supported by NIH/NIDCR grant 1 R21 DE017673-01A1, the Juvenile Diabetes Research Foundation and the University of Kansas General Research Fund.

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Received: May 23, 2008
Accepted: June 23, 2008