Cell Interactions with Biomaterials Gradients and Arrays[§]

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Abstract: Gradients and arrays have become very useful to the fields of tissue engineering and biomaterials. Both gradients and arrays make efficient platforms for screening cell response to biomaterials. Graded biomaterials also have functional applications and make useful substrates for fundamental studies of cell phenomena such as migration. This article will review the use of gradients and arrays in tissue engineering and biomaterials research, with a focus on cellular and biologic responses.

Keywords: Biomaterials, cell adhesion, cell-material interactions, combinatorial screening, gradient, hydrogel, microarray, polymer, tissue engineering.

1. INTRODUCTION

Despite significant investment in tissue engineering research, few profitable products have come to market [1, 2]. Hence, there is a need to accelerate tissue engineering research. One approach to accelerating research is combinatorial and high-throughput screening (CHT) (see Table **1** for all abbreviations used herein). Traditional research involves preparing samples one at a time for characterization and testing. In contrast, with CHT approaches, libraries are fabricated that combine many samples into miniaturized specimens. These libraries lower the cost of research by reducing the amount of time and material required for experiments [3]. Combinatorial approaches are utilized extensively for pharmaceutical research [4, 5] and their utility in biomaterials research is becoming apparent [6].

CHT methods for biomaterials research use two types of specimens: continuous gradients and discrete arrays (Figs. **1**, **2**). Gradients involve specimens that have continuously changing properties (composition, ligand density) along one or more of their axes. Arrays involve small, discrete specimens placed closely together on the same substrate (96-well plate, glass slide). There are advantages and disadvantages to both gradients and arrays (Table **2**). Arrays can be easier to characterize but only include selected compositions. Plus, individual sample handling is required for each composition. Gradients require more character-

Table 1. Table of Abbreviations

2D	two-dimensional
3D	three-dimensional
bFGF	basic fibroblast growth factor
bisGMA	2,2-bis[4-(2-hydroxy-3- methacryloxypropoxy)phenyl] propane
BMP-2	bone morphogenetic protein-2
CBFA1	core binding factor alpha 1
CHT	combinatorial and high-throughput
ECM	extracellular matrix
EGMP	ethylene glycol methacrylate phosphate
FGF-2	fibroblast growth factor-2
IGF-II	insulin-like growth factor-II
IKVAV	isoleucine-lysine-valine-alanine-valine peptide
NGF	nerve growth factor
NT-3	neurotrophin-3
PCL	poly(ε-caprolactone)
PDLLA	poly(D,L-lactic acid)
pDTEc	poly(desaminotyrosyl-tyrosine ethyl ester carbonate)
pDTOc	poly(desaminotyrosyl-tyrosine octyl ester carbonate)
pHEMA	poly(hydroxyethyl methacrylate)
PLGA	poly(lactic-co-glycolic acid)
PLLA	poly(L-lactic acid)
SEM	scanning electron microscope
TEGDMA	triethylene glycol dimethacrylate

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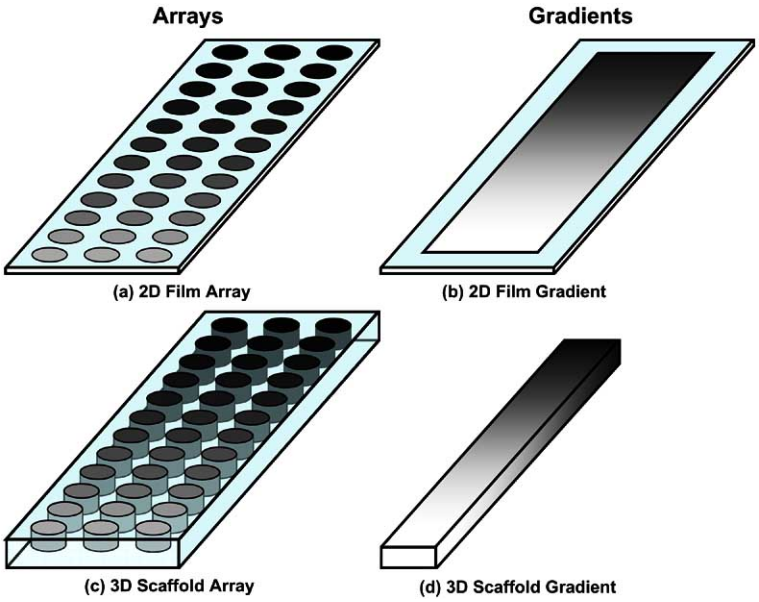

Fig. (1). Diagrams of (**a**) 2D gradient, (**b**) 2D array, (**c**) 3D scaffold gradient and (**d**) 3D scaffold array. 2D means the materials are presented as 2D flat films, surfaces or spots. 3D means the materials are presented as 3D scaffolds.

ization, but contain all possible compositions in a single specimen. Sample handling is significantly reduced for gradients since only one specimen is required. Arrays are amenable to *quantitative* analyses since individual assays can be performed for each discrete composition. Gradients, on the other hand, are amenable to more rapid, *qualitative* assessment since an entire composition range can be tested in a single specimen. Lastly, data analysis is usually easier for arrays than for gradients.

It is also important to keep in mind that cells rarely interact directly with a biomaterial. Proteins present in blood *in vivo* or serum *in vitro* immediately adsorb onto most materials. Thus, cell response to a biomaterial is strongly influenced (possibly dominated) by the species, amount and conformation of proteins that adsorb onto a biomaterial [7]. Note that the current review focuses on biomaterials and that a large literature exists where gradients of factors have been fabricated and examined for their roles in development and cell migration. Though this work is partially covered here, these topics are beyond the scope of the current review [8-13].

2. TYPES OF GRADIENTS AND ARRAYS: CLASSIFICATION

One way to classify gradients and arrays that have been used to examine cell response to biomaterials is by physical structure. The simplest structural delineation is two-dimensional (2D) versus three-dimensional (3D) (Figs. **1**, **2**). The majority of published work with biomaterials gradients arrays and cell screening has been done in a 2D format, i.e. films and surfaces (Table **3**). For arrays, an array of biomaterial "spots" or "wells" in the form of films or surfaces with varying properties are presented to cells and cell function is assayed. For gradients, a substrate containing a biomaterial film, hydrogel or surface containing a gradient in properties is presented to the cells and cell response is observed.

For 3D systems, cells are seeded *within* a hydrogel (not *on* the hydrogel) or onto a large-pore scaffold (> 0.1 mm dia. pores) such that the cells can explore the test biomaterials in three dimensions (Table **3**). For arrays, an array of miniature scaffolds with varying properties would comprise the library. For gradients, a hydrogel or large-pore scaffold containing a continuous gradient in properties would comprise the library.

3. 2D FILMS & SURFACES

3.a. 2D Gradients: Surface Energy

Surface energy is a fundamental material property that can influence cell behavior. Substrates with gradients of surface energy were some of the first gradients used to probe cell-material interactions [14-28]. Several methods for fabricating surface energy gradients employing a variety of surface chemistries have been demonstrated (reviewed in [19]). In addition, many cell functions have been studied during culture on surface energy gradients: adhesion, adhesion strength, morphology, spread area, migration, proliferation and differentiation. Collectively, the results from these studies do not always agree with one another.

For example, cell adhesion is enhanced on different surface energies in different reports. Cell adhesion is equal on all of the water contact angles in Ruardy *et al.* [14], adhesion is enhanced on the hydrophobic regions in Ueda-Yukoshi & Matsuda [15] while adhesion was highest in hydrophilic regions in Chaudhury *et al.* [27]. Each study used a different cell type which suggests that cell adhesion can vary between cells. In addition, the surface chemistries used to make the gradients are different from each report. Differences in cell types and surface chemistry may explain the different observations made in these studies. One firm conclusion from these investigations is that surface energy can influence cell function.

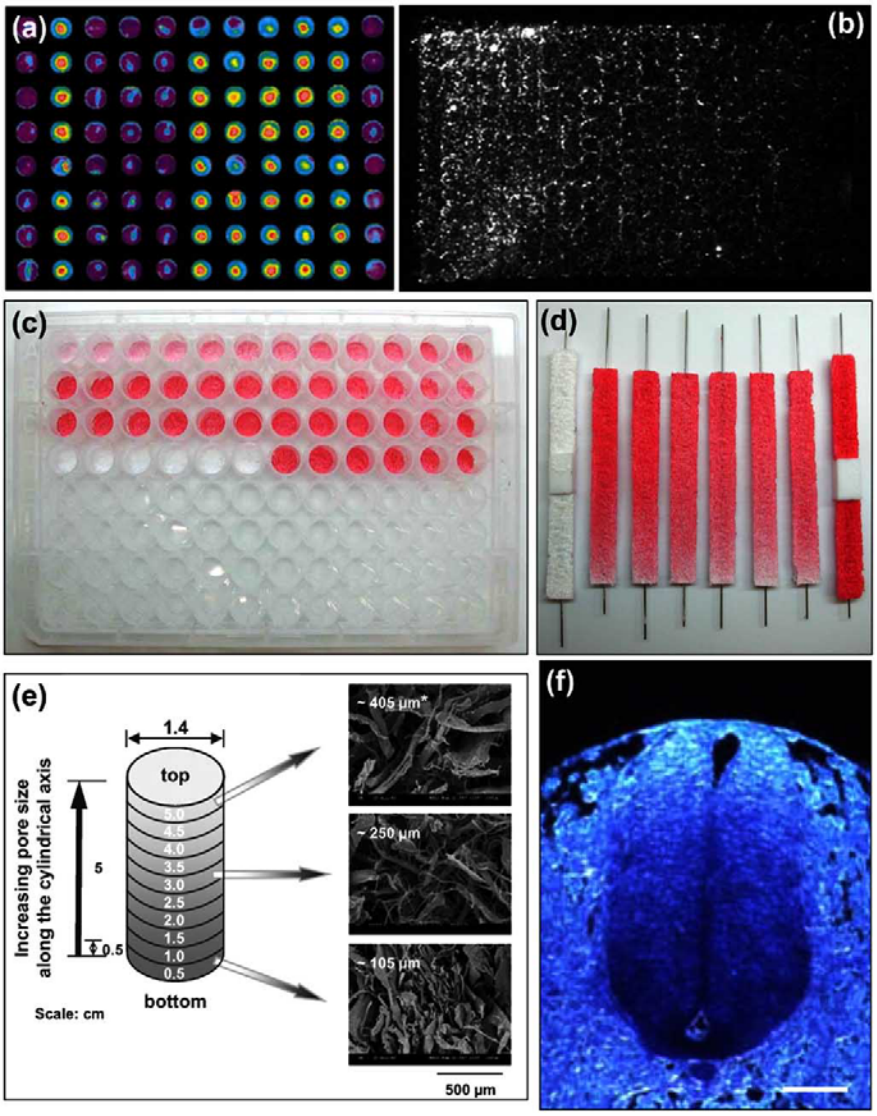

Fig. (2). Examples of different types of gradients and arrays from the literature. (**a**) 2D Surface Array: Fluorescently-labeled primary chicken hepatocytes cultured on glycan arrays are visualized by fluorescence microscopy (used with permission [67]). Each column of spots is a different glycan, red indicates enhanced cell adhesion and each spot is 1.8 mm in diameter. (**b**) 2D Surface Gradient: A surface gradient of biotinylated-FGF-2 was fabricated using an inkjet printer and visualized with streptavidin-conjugated-quantum dots (used with permission [40]). The gradient is 1.75 mm by 1.25 mm. (**c**) 3D Scaffold Array: An array of salt-leached polymer scaffolds with varying composition fabricated in a 96-well plate (used with permission [92]). (**d**) 3D Scaffold Gradient: Rod-shaped, salt-leached, polymer scaffolds with a gradient in composition [92]. Six gradients 75 mm long, 8 mm wide and 4 mm deep are shown. Controls are shown on the sides. (**e**) 3D Pore Size Gradient: Polymer scaffold with a gradient in pore size created by a centrifugation method (used with permission [105]). Average pore size is given in the SEM images. (**f**) Gradients Exist *In Vivo*: Gradient in netrin-1 protein in the developing spinal chord of a day 9 mouse embryo visualized by immunohistochemistry (used with permission [115]). Size bar is 0.05 mm.

3.b. 2D Gradients

Cell response to biomaterial gradients has been most frequently studied in "2D" systems where cell response to a gradient is assessed during culture on 2D films and surfaces [29-64]. Many innovative approaches have been employed to fabricate these gradients, and a variety of material parameters have been explored including gradients in i) proteins (cell membrane preparations [30]; basal lamina extracts [31]; SemA and SemC [32]; collagen I [41]; fibronectin [35, 38, 40, 43, 48, 49, 63]; laminin [31, 33, 41, 42, 46]; FGF-2 [40, 62] (Fig. **2b**); ephrinA5 [50]; IGF-II [62]; BMP-2 [62]), ii) peptides (RGD from fibronectin [37, 47, 51]; IKVAV from laminin [36]; B160 peptide from laminin [56]; A10 peptide from laminin [53]), iii) polymer composition (PCL:PDLLA [34, 59]; PDLLA:PLLA [44]; PCL:PGLA [45, 64]; dental resins [58, 60]; pDTEc:pDTOc [61]) and iv) material processing (polymer crystallinity [39]; dental resin conversion [57, 58, 60]; roughness [52, 55, 56, 61]).

Table 3. Biomaterial Properties Varied in Gradients and Arrays

2D Gradients	Surface energy [14-28], cell filtrate or serum gradient [29], counter gradients of anterior and posterior tectal cell membranes [30], basal lamina protein gradients & merosin gradient [31], Sema3A & Sema3C gradient [32], albumin/laminin gradient [33], PCL/PDLLA composition/annealing gradient [34, 59], fibronectin gradient [4, 35, 38, 43, 48, 63], IKVAV laminin peptide gradient [36], RGD gradient [37], PLLA crystallinity gradient [39], FGF-2 gradient [40], laminin/coll I gradient [41], laminin gradient [42, 46], PDLLA/PLLA gradient [44], PLGA/PCL composition/annealing gradient [45, 64], RGD gradient [47, 51], ephrinA5 gradients [50], silica nanoparticle density gradient [52, 56], laminin B160 peptide gradient [53], laminin A10 peptide gradient [54], roughness gradient [55], dental resin bisGMA/TEGDMA composition/cure gradients [57, 58, 60], pDTEc/pDTOc composition/annealing gradients [61], immobilized BMP-2, IGF-II and FGF-2 gradients [62]
2D Arrays	Acrylate-based polymer arrays [65], carbohydrate arrays [66, 67], polyarylate polymer array [68], polymer array [69, 72, 74, 77], ECM protein array [70, 71], growth factor array [73], polymer blend array [75], polyanhydride array [76]
2D Hydrogel Gradients : Cells *on* Gels	modulus gradient [78], RGD gradient [79], NGF gradient [80], RGD gradient [81], bFGF gradient [82]
2D Hydrogel Arrays : Cells *on* Gels	fibronectin/modulus array [83], polymer array [84]
3D Scaffold Gradients	scaffold surface treatment gradient [90], covalently-linked protein gradient [91], polymer composition gradient [92], nanofibers with laminin gradient [93]
3D Scaffold Arrays	polymer composition array [92], pDTEc/pDTOc array [94]
3D Hydrogel Scaffold Gradients: Cells *in* Gels	laminin gradient [95], NGF and NT-3 concentration gradient [96], RGD gradient [97], laminin-1 and NGF gradient [116]
3D Hydrogel Scaffold Arrays: Cells *in* Gels	*(none to our knowledge)*
3D Scaffold Pore Size/Porosity Gradients	porosity gradient [101, 102], pore size/porosity gradient [103, 105], pore size gradient [104]
3D Scaffold Pore Size/Porosity Arrays	*(none to our knowledge)*

In many studies, one material parameter is varied across the gradients [29, 31, 35-40, 42, 46, 47, 49, 51-57, 62, 63]. However, counter gradients have been fabricated where two components are varied in opposite directions from one another (cell membrane preparations [30]; SemA and SemC [32]; laminin/albumin [33]; PCL/PDLLA [34, 59]; laminin/ collagen I [41]; pHEMA/fibronectin [43, 48]; PLLA/PDLLA [44]; PLGA/PCL [45, 64]; bisGMA:TEGDMA [58]; BMP-2/FGF-2 [62]). In addition, libraries have been fabricated where two parameters are varied orthogonally from one another: polymer composition and annealing temperature [34, 45, 59, 61, 64]; polymer composition and degree of polymerization [58, 60]. Also, the effect of the slope of biomaterial gradients on cell function has been explored in a number of studies [33, 46, 49, 54]. Thus, one parameter gradients, 2 parameter counter gradients, 2 parameter orthogonal gradients and gradients with different slopes have been used to examine cell behavior.

In general, increased surface concentration of extracellular matrix proteins (ECM) proteins (laminin, fibronectin), adhesive peptides (RGD, IKVAV) or growth factors (FGF-2, BMP-2) in the gradients correlates with enhanced cell functions such as adhesion, spreading, proliferation, migration and differentiation [33, 35-38, 40-42, 47, 49, 51, 62]. In some cases, there is a threshold for surface density above which there is no increased effect on cell response [46, 51] or the cell response is inhibited [43, 48, 53, 54]. Several different observations were made when the slopes of protein or peptide gradients were varied: 1) cell functions were enhanced with increasing slope [49, 54], 2) cell functions were not affected by slope [32, 46], 3) there was a threshold for the slope below which the cells did not respond to the gradient and above which the cell response was enhanced by the gradient [33, 50] or 4) cell functions were enhanced with decreasing slope [30, 50].

Several cell studies with gradients of polymer composition or processing variables have been reported. For PLLA films annealed on a temperature gradient stage, the cool end stays smooth and amorphous while the hot end crystallizes and becomes rough [39]. Cell proliferation increased linearly with increasing PLLA crystallinity and roughness on these gradients. For composition gradients of PDLLA and PLLA, cell proliferation was enhanced only on the most PDLLA-rich regions [44]. Orthogonal gradients of polymer composition and annealing temperature induced polymer phase separation that yielded libraries with wide variations in domain sizes, roughness and composition. The cell culture results for these systems were especially interesting because unique composition/temperature combinations were observed to enhance cell functions [34, 45, 59, 61, 64]. These unique combinations may not have been discovered if discrete specimens had been used instead of the gradient approach.

For micron-scale roughness gradients made by sandblasting, opposite effects were found for different cell types. Osteoblasts showed increased proliferation rate with increasing surface roughness while fibroblasts had decreased proliferation with increasing roughness [55]. For nano-particle gradients made by adsorbing silica nanoparticles to substrates, increased particle surface coverage inhibited cell proliferation [52, 56]. Finally, the viability of cells cultured on conversion gradients of a photopolymerizable dental resin was enhanced with increasing resin conversion [57, 58, 60].

Thus, many creative approaches for screening cell response to biomaterials using films or surfaces containing gradients have been developed.

3.c. 2D Arrays

"Two-dimensional" biomaterial arrays, where materials are presented to cells as discrete, flat films, surfaces or spots, have also been used to test cell response [65-77]. Several of these platforms used well-plates as a platform for biomaterial films [68, 75-77] while others used robotics to "spot" the biomaterials onto flat substrates (i.e. glass slides, Petri dishes) [65-67, 69, 70, 71, 73] (Fig. **2a**). These arrays have covered several types of biomaterials including polymers [65, 68, 69, 72, 74-77], proteins [70, 71, 73] and carbohydrates [66, 67].

The studies with 2D *polymer* arrays have taken a number of innovative perspectives. Smith *et al*. [68] observed significant differences in fibroblast proliferation and macrophage expression of inflammatory cytokines during culture on arrays of degradable polyarylates. They used the large volume of data collected to successfully validate an artificial neural network algorithm for predicting cell response to untested materials. Anderson *et al*. [65] synthesized a polymer library of 576 different materials by photopolymerizing spotted blends of various acrylate monomers in situ. Most of the polymers supported human embryonic stem cell adhesion and differentiation into epithelia, however, several did not. In a follow-up, Anderson *et al*. [69] spotted blends of 24 different polymers into 1152 different ratios and combinations. Human mesenchymal stem cells adhered and spread on most blends although some combinations, especially those containing poly(ethylene glycol), inhibited attachment. Mant *et al*. [72] and Tourniaire *et al*. [74] synthesized a library of 120 different polyurethanes and spotted them onto agarose coated glass slides. The arrays were used to screen cell adhesion proliferation, and several hits were identified using either primary mouse bone marrow dendritic cells or primary human renal tubular epithelial cells. All of the "hits" contained 4,4'-methylenebis (phenylisocyanate) indicating that inclusion of this diisocyanate can enhance cell adhesion.

Two companies have used biomaterial arrays for hemocompatibility screening. Cawse *et al*. [75] exposed a biomaterials library array to blood and found significant differences in leukocyte activation and platelet adhesion. In addition, Hezi-Yamit *et al*. [77] screened arrays of polymers designed for stents and found significant differences in monocyte adhesion and activation. Adler *et al*. [76] synthesized a library of polyanhydrides by using robotics to mix monomers in different ratios and found that several of the polymers were cytotoxic and induced an inflammatory response in cells cultured *in vitro*. Thus, arrays of polymeric biomaterials have been fabricated in many formats and screened for a variety of cellular behaviors.

A handful of studies have screened cell interactions with 2D arrays of naturally-occurring biomaterials: carbohydrates [66, 67] and proteins [70, 71, 73]. Disney *et al*. [66] spotted 5 different monosaccharides (glucose, mannose, galactose, fucose, *N*-acetylglucosamine) over a 1000-fold range of concentrations and found that *E. coli* adhered only to the mannose spots and that the adhesion increased with mannose concentration. Nimrichter *et al*. [67] screened arrays containing 45 different mono- and oligosaccharides and observed that hepatocytes adhered to glycans with terminal *N*-acetylglucosamine but not to glycans with terminal galactose or *N*-acetylgalactosamine (Fig. **2a**). For primary human T-cells, Nimrichter *et al*. [67] found the best adhesion to the sialyl Lewis x structure.

Flaim *et al*. [70] spotted 32 combinations of 5 ECM proteins (collagen I, collagen III, collagen IV, laminin, fibronectin) into arrays and measured albumin expression by primary rat hepatocytes as a marker for maintenance of a differentiated phenotype. Albumin expression was highest in spots containing collagen IV, fibronectin also positively affected albumin expression and laminin and collagen III negatively affected albumin expression. Kuschel *et al*. [71] spotted 16 different ECM proteins into arrays on slides and found many differences in adhesion for several cell lines, though fibronectin and tenascin were the best and worst in all cases, respectively. Soen *et al*. [73] spotted laminin with combinations of 14 different signaling molecules onto slides and examined neural precursor differentiation down neural or glial fates. Four outcomes were observed: 1) neurogenesis, 2) gliogenesis, 3) undifferentiated, and 4) both.

Comparison of these array technologies is insightful. Platforms that contain the biomaterials in individual wells enable quantitative measurement of cell responses using colorimetric assays [68, 75-77]. For biomaterials arrayed together on a single flat substrate, qualitative or semi-quantitative microscopy-based evaluation must be used for evaluating cell response [65-67, 69-71, 73]. On the other hand, the approaches where the materials are spotted together enables a larger degree of miniaturization where more samples can be packed into one array specimen, up to 3456 spots per slide in the case of Anderson *et al*. [69]. Another consideration is paracrine signaling where cells on one spot could secrete factors that could diffuse to cells on a neighboring spot. Paracrine signaling is also a concern for gradient libraries. However, paracrine signaling cannot occur if "spots" are contained in separate wells of an array. In sum, these innovative studies elegantly demonstrate how synthetic polymers and natural biomolecules can be arrayed and screened for their effects on cells.

3.d. 2D Hydrogel Gradients: Cells *on* Gels

Next, examples of screening cell response during 2D culture *on* hydrogels containing gradients will be discussed (3D culture *in* hydrogels gradients will be addressed later) [78-82]. Lo *et al*. [78] varied hydrogel crosslinking density to create modulus gradients and found that fibroblast migration and spreading were enhanced with increased modulus. Burdick *et al*., [79] made hydrogels with concentration gradients of tethered RGD and found better adhesion and spreading of endothelial cells with increasing RGD. Kapur *et al*. [80] varied the slope of immobilized NGF concentration gradients and observed that neural cell migration up the gradients was enhanced only on NGF gradients with the steepest slope.

Delong *et al*. [81] cultured human dermal fibroblasts on hydrogels with surface gradients of RGD peptide and found that the cell extensions aligned with the gradients and that

the cells tended to migrate up the gradients. Finally, DeLong *et al*. [82] synthesized hydrogels with bFGF gradients and observed that aortic smooth muscle cells aligned in the direction of the gradients and migrated up the gradients towards increasing bFGF. These reports show how hydrogel gradients can be used to screen the effect of substrate stiffness, biomolecule concentration and gradient slope on cell performance.

3.e. 2D Hydrogel Arrays: Cells *on* Gels

Hydrogel arrays have been used to screen cell response using 2D culture of cells *on* gels [83, 84] (to our knowledge). Semler *et al*. [83] varied two hydrogel properties orthogonally to one another in an array format in a 96-well plate: hydrogel stiffness and fibronectin concentration. Hepatocytes displayed increased spreading with increasing modulus and fibronectin while albumin secretion (marker for the differentiated phenotype) was enhanced with decreasing modulus. These results neatly demonstrated how a combinatorial approach enables screening of multiple material parameters. Benoit *et al*. [84] photocured robotically spotted combinations of monomers with different chemical functionalities onto flat substrates. They found that the different functionalities stimulated human mesenchymal stem cells to differentiate down different lineages: ethylene glycol methacrylate phosphate (EGMP) containing gels enhanced CBFA1 expression (marker for osteoblasts); methacrylic acid enhanced aggrecan expression (marker for chondrocytes); *tert*-butyl methacrylate and 2,2,3,3 tetrafluoropropyl methacrylate containing gels enhanced expression of PPARG (peroxisome proliferative activated receptor gamma, marker for adipocytes). These results are especially significant because they demonstrate that stem cell differentiation can be guided through interactions with synthetic biomaterials.

4. 3D: SCAFFOLDS AND HYDROGELS

4.a. 3D Scaffold Gradients

Most CHT work with biomaterials has been done with 2D surfaces and films, however, biomaterials are commonly used in a three-dimensional (3D) scaffold format [85, 86] and cells behave more physiologically when cultured in 3D [87-89]. Thus, recent studies have focused on CHT methods where the materials are presented to cells in a 3D scaffold format [90-93]. Work from our own group has introduced a method for making gradient scaffold libraries where scaffold composition can be varied [92] (Fig. **2d**) and future work will apply the scaffold library approach for screening cell response. Barry *et al*. [90] used plasma polymer deposition to fabricate functional scaffolds containing a gradient. A thicker layer of polymer was deposited on the scaffold periphery than in the scaffold core leading to more even cell distribution in the scaffolds upon cell seeding. Vepari *et al*. [91] have demonstrated a method for fabricating scaffolds containing a gradient in covalently-linked protein and plan to apply this method to create graded tissues. Valmikinathan *et al*. [93] fabricated electrospun polymer fiber gradients containing a gradient in laminin and found that cell adhesion was enhanced with increasing laminin.

The focus of future CHT may shift more to scaffolds due to the potential for more physiological cell behavior during cell culture in 3D. However, CHT screening of scaffolds will present new challenges because 3D scaffolds are harder to study than 2D surfaces. Scaffolds are harder to fabricate, require more material, harder to characterize and harder to seed and culture with cells. It is also more challenging to measure cell response after culture in 3D scaffolds than after culture on 2D surfaces.

4.b. 3D Scaffold Arrays

Our group has also developed a method for fabricating combinatorial scaffold arrays [92] (Fig. **2c**) and used it for screening the effect of scaffold composition on cell response [94]. A binary blend system of degradable polycarbonates (pDTEc & pDTOc) was screened and cell adhesion and proliferation were enhanced with increasing pDTEc composition in the scaffolds.

4.c. 3D Hydrogel Scaffold Gradients: Cells *in* Gels

Methods for examining cell response during culture *in* gradient hydrogels (as opposed to culture *on* gradient hydrogels) have also been demonstrated [95-97]. Dodla *et al*. [95] cast dorsal root ganglia in agarose gels and used diffusion to establish laminin gradients (6 h). The laminin gradients were immobilized by photochemistry and neurite outgrowth was monitored for 4 d. The presence of laminin gradients in the hydrogels significantly enhanced the rate of neurite extension from the cells. Moore *et al*. [96] used a gradient maker to cast pHEMA gels containing gradients in NGF and NT-3 which were immobilized during photocrosslinking of the pHEMA. Dorsal root ganglia cells were seeded on top of gels and during culture penetrated into the gels. The cells extended neurites up gradients only when gradients of both factors were present and not in the presence of a gradient of either factor alone, suggesting a synergistic effect. Musoke-Zawedde and Shoichet [97] used UV laser micropatterning to fabricate RGD peptide gradients in hyaluronan gels and observed that the gradients guided neurite outgrowth from primary neural cells. These studies nicely demonstrate 3D methods for studying cell response using gradients.

4.d. 3D Hydrogel Scaffold Arrays: Cells *in* Gels

Methods for fabricating hydrogel arrays with uniform gel composition have been described [98-100], but none have yet been described where gel composition is varied in the arrays for screening the effect of hydrogel composition on cell response (to our knowledge).

4.e. 3D Scaffold Pore Size/Porosity Gradients

Several techniques have been reported for fabricating scaffolds containing gradients in pore size or porosity [101-105]. A common motivation for this work has been mimicry of the graded tissue morphologies present *in vivo* [101-104]. In order to mimic the transition zones between cortical (non-porous) and spongy (porous) bone found *in vivo*, Tampieri *et al*. [101] implanted ceramic scaffolds containing a gradient in porosity into rabbit femur defects. New bone formation was increased in the higher porosity regions of the scaffolds. Aiming to mimic the porosity gradients present in spongy bone *in vivo*, Roy *et al*. [102] used freeform fabrication to fabricate polymer-ceramic composite scaffolds containing porosity gradients. When implanted in rabbit calvarial

defects, more new bone was formed in the high porosity zones than in the low porosity zones of the scaffolds. Woodfield *et al*. [103] also used freeform fabrication to create polymer scaffolds with pore size/porosity gradients. Chondrocytes seeded on the constructs produced a layered cartilage matrix that resembled the zonal organization of native articular cartilage with respect to cell density, glycosaminoglycan and collagen II. Finally, Hoffman *et al*. [104] demonstrated that silk scaffolds with pore size gradients will induce formation of a tissue with a graded morphology.

Only one study explicitly fabricated scaffolds with a pore size/porosity gradient for the intent of screening the effect of pore size/porosity on biological response [105] (Fig. **2e**). Chondrocytes and osteoblasts formed the most tissue in large pore/high porosity scaffolds while fibroblast numbers were higher on smaller pore size/lower porosity scaffolds. Bone formation after implantation into rabbit calvarial defects was highest for mid-range pores size/porosity scaffolds.

4.f. 3D Scaffold Pore Size/Porosity Arrays

No methods for fabricating arrays of scaffolds with varied pore size/porosity have been reported (to our knowledge).

5. DISCUSSION

5.a. Graded Biomaterials: Gradients *In Vivo*

Gradients are present in many forms *in vivo* presenting rich opportunities for functional application of graded biomaterials [106]. As mentioned previously, porosity gradients are present in bones where cortical bone transitions into spongy bone [101, 104, 107]. Articular cartilage has a zonal organization with differential protein expression and collagen fiber alignment in the different layers [103, 108, 109]. A tissue gradient exists at the ligament to bone interface where ligament transitions to fibrocartilage which transitions into bone [110, 111]. Morphogen gradients are present during development and direct cell proliferation [112], tissue generation [11, 113] and nervous innervation [114, 115] (Fig. **2f**). In crypt villi, the functional units of the small intestine, the composition of the basement membrane changes in a graded fashion as you move up the villi towards the absorptive cells [116]. Teeth contain gradients in composition and mineral density giving rise to gradients in mechanical properties [117]. Gradients in composition and properties are common *in vivo*, especially at boundary zones between different tissues, and the need for "interface tissue engineering" is well-recognized. Thus, graded biomaterials are being used as templates for the generation of graded tissues and can guide cell migration or axon growth *in vivo* [101-104, 107-110, 115].

5.b. Data Analysis and Modeling

CHT methods yield large data sets amenable to advanced data analysis and modeling and several reports highlight these approaches. Kohn's group (Rutgers University) has used the cell response data collected using their combinatorial arrays of polyarylates to test and validate an artificial neural network that accurately predicted cell behavior [68, 118]. They also used this CHT data set to develop a partial least squares regression approach, commonly used by the pharmaceutical industry in quantitative structure activity relationship analysis [6, 119]. Predictions from this model were also very accurate and have the potential to enable and guide rational biomaterials design by synthetic polymer chemists. In addition, Liu *et al*. [60] used automated microscopy and data analysis to collect large sets of cell morphology data from polymer gradients. Bivariate correlation coefficients were computed and it was demonstrated that they can be used to identify correlations between cell morphology descriptors and material properties. Finally, Meredith's group (Georgia Tech.) has employed advanced methods to analyze large volumes of cell data collected on polymer composition-annealing gradients [59, 64]. They developed a method for correlating an individual cell's behavior with its distance from particular morphological features present in the material libraries. The new analytical method, termed "local cell feature analysis", provided unique, instructive insight into the effects of biomaterial surface morphology on cell performance which was not observed using traditional analysis methods [64]. As CHT methods for screening cell-biomaterial interactions mature, these types of improved approaches for data analysis will become increasingly important.

6. FUTURE DIRECTIONS

The creativity and innovation in the application of CHT methods to screening cell-biomaterial interactions has been astounding and it is exciting to consider where the field will go in the future. One especially intriguing possibility is CHT *in vivo* screening. Miniaturized biomaterial arrays or gradients could be used in animal models for rapidly screening *in vivo* performance of biomaterials. A large number of samples present in a combinatorial specimen could be tested in a single animal, reducing the cost, time and number of animals required for *in vivo* screening of biomaterials. It will also be intriguing to watch CHT biomaterial platforms translate from academic research into industrial practice. If biomaterial gradients and arrays can truly accelerate the development of new biomaterials, then their commercial use should be on the rise.

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