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REVIEW ARTICLE

TERM

Synthetic Hydrogels with Stiffness Gradients for Durotaxis Study and Tissue Engineering Scaffolds

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Migration of cells along the right direction is of paramount importance in a number of *in vivo* circumstances such as immune response, embryonic developments, morphogenesis, and healing of wounds and scars. While it has been known for a while that spatial gradients in chemical cues guide the direction of cell migration, the significance of the gradient in mechanical cues, such as stiffness of extracellular matrices (ECMs), in directed migration of cells has only recently emerged. With advances in synthetic chemistry, micro-fabrication techniques, and methods to characterize mechanical properties at a length scale even smaller than a single cell, synthetic ECMs with spatially controlled stiffness have been created with variations in design parameters. Since then, the synthetic ECMs have served as platforms to study the migratory behaviors of cells in the presence of the stiffness gradient of ECM and also as scaffolds for the regeneration of tissues. In this review, we highlight recent studies in cell migration directed by the stiffness gradient, called durotaxis, and discuss the mechanisms of durotaxis. We also summarize general methods and design principles to create synthetic ECMs with the stiffness gradients and, finally, conclude by discussing current limitations and future directions of synthetic ECMs for the study of durotaxis and the scaffold for tissue engineering.

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Key Words: Durotaxis; Mechanical properties; Synthetic hydrogel; Tissue engineering

INTRODUCTION

Cells residing on or within *in vivo* extracellular matrices (ECMs) experience multiple signaling cues in the form of soluble or matrix-bound chemical species, tension and compression applied via matrix by external forces or neighboring cells, stiffness or topographic feature of matrix, and so on. When there exists a spatial variation, or a gradient, in the level of a signaling cue that extends over the cell body, the cell is known in general to be polarized and to migrate toward the region with a higher level of cue. Such directed migration of cells induced by a gradient in, typically, the concentration of soluble chemoattractants, the density of matrix-bound chemoattractants (typically, cell adhesion ligands), or matrix stiffness is called chemotaxis, haptotaxis, or durotaxis, respectively [1], and is suggested to be an essential process in immune response [2,3] (e.g., neutrophils chasing and killing pathogens), healing of wounds and scars [4-6] (e.g., fibroblasts or stem cells trafficking to the sites of wounds and fibrotic scars), embryonic development and morphogenesis [7-9], metastasis of cancer cells [10-12], or progressions of atherosclerosis and liver fibrosis [13-15].

While directed migration of cells in response to gradients in chemical cues has been studied up to tens of decades ago, the first experimental descriptions of chemotaxis being in 1881 [16, 17] and haptotaxis in 1967 [18], the effects of gradients in mechanical cues, for example stiffness, on the directed migration of cells and their mechanisms have been explored only recently, the term durotaxis was first coined in 2000 [19]. Gradients in stiffness exist *in vivo* within or at the interfaces of ECMs and tissues and are found to direct the migration of cells during wound healing, pathogenesis, development, and so on [5,6,20-23]. For example, contractions of myofibroblasts at the wound site stiffen neighboring ECMs [23]; breast cancer cells are found to be almost 10 times stiffer than distant normal tissues [24]; a fibrotic scar formed in myocardium post-infarction is stiffer than surrounding tissues by a factor of three to four [25]; or stiffness increases drastically at the interface between calcified bones and connected soft cartilage [25-27].

Developments in characterization methods enabling microscopic measurements of local deformation or stiffness, such as traction-force microscopy [28,29] or nano-indentation using atomic force microscopy [30-32], as well as synthetic chemistry

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and micro-fabrication techniques have facilitated the *in vitro* study of durotaxis by aiding a creation of synthetic ECMs with microscopic patterns of stiffness and a quantification of the cell-generated traction force, which is found to be related to the local stiffness of ECMs, at a length scale as small as a few micrometers. Synthetic ECM with controlled stiffness-gradients can serve not only as a platform for studying durotaxis *in vitro* but also as a scaffold to induce desired cellular behaviors and thus to guide tissue formation [33-36], provided that the scaffold meets the general requirements of 3D cell culture [37]; i.e., cell-mediated remodeling of matrix and adequate levels of nutrients, oxygen, and adhesion ligands. In fact, a spatiotemporal control of matrix mechanics has begun to be incorporated in tissue engineering strategies to guide the behaviors of encapsulated cells, including morphology, proliferation, and differentiation at right positions and timing [38-40]. For a better understanding of cellular durotaxis and for a successful regeneration of tissue, therefore, an appreciation of design principles of recapitulating mechanical cues of *in vivo* microenvironments in synthetic ECMs is of paramount importance.

In this review, we first briefly discuss the mechanisms of durotaxis investigated up to date and then summarize general methods to create synthetic ECMs, mostly hydrogels, with gradients, or spatiotemporal variations of stiffness. Next, we introduce by cell types *in vitro* studies of durotaxis carried out using synthetic ECMs. Finally, we discuss current limitations and future directions of synthetic ECMs for the study of durotaxis and as the scaffold for tissue engineering.

MECHANISMS OF DUROTAXIS

The process by which adherent cells migrate along the stiffness gradient of underlying ECM is understood to begin with a protrusion at the front of the cell where the polarity-front or back-of the cell is determined by the arrangement of microtubules and/or the receptor on the cell surface. It is found that such polarity is more stable for cells undergoing a directed migration (e.g., chemotaxing) than those meandering in a random way with their polarities being continually switched [41,42]. The protrusion of the cell front is driven by the growth of actin filaments that push the cell membrane forward [43-45]. When Arp (Actin-related protein) 2/3 complex binds to an existing actin filament in response to Rac1 GTPase activated by the external stimuli, the complex provides a nucleation site for the polymerization of a new actin filament. While actin monomer continues to assemble at one end of the filament to push the cell membrane forward, the opposite end of the filament is disassembled and severed by actin-depolymerizing factor (ADF) and cofilin. Such dynamic cellular structure generated by the tread-milling mesh of actin filament at the cell front is called lamellipodia.

When lamellipodia protrudes, adhesion receptors on the cell membrane such as integrin are linked to adhesion ligands of ECM, which initiates intracellular signaling cascade to form multi-protein complexes that connect ECM ligands to the cytoskeleton. Such adhesion complexes consisting of a few to over a hundred proteins [46-48] are dynamically assembled, disassembled, or remodeled and act as "molecular clutch" that engage or disengage the transmission of forces between ECM and the cell [49]. Adhesion complexes are nascent within lamellipodia but grow into mature ones called focal adhesion complexes (FAC) within lamella, which is the cellular structure located behind lamellipodia and is responsible for the cell motility [43,50].

FAC is linked to stress fibers, which are bundles of cross-linked actin filaments connected to each other via myosin motor proteins. Myosin catalyzes the hydrolysis of ATP to generate the contractile force of stress fibers (actomyosin contractility), which pulls the connected FAC toward the center of the cell. Such retrograde flow of actin filaments at the cell front is translated into the traction force applied to ECM via FAC, which enables the cell to migrate forward. On the other hand, FAC also transmits forces generated by ECM to cytoskeleton, for example the resistance of ECM against deformation by the cell traction force or stretching or bending occurring during physiological processes such as breathing. In this respect, FAC can be considered as the molecular clutch that transmits forces between the cell and ECM (Fig. 1).

Transmission of endogenous or exogenous mechanical forces results in the alteration of individual structure or arrangement of mechanosensitive proteins of FAC such as talin, vinculin, and p130Cas and actomyosin [46,51-54], for example by stretching the protein conformation to expose hidden peptide sequence [52]. Such conformational changes may trigger intracellular signal cascades that results in changes in the dynamic structures of FAC and stress fibers through the assembly, disassembly, recruitment, or rearrangement of various proteins associated with FAC and stress fiber. The changes in the dynamic structures also result in the changes in the mechanical strength of FAC and stress fiber as exemplified by their stress-stiffening behaviors, the lifetime of the bond between ECM ligand and integrin due to their catch-bond characteristics, or the intracellular flux of calcium ions through the stretch-activated ion channels located at or near FAC [52-54].

The integrated response of the cell to the ECM-generated force that is transmitted via FAC and stress fibers is to adjust its endogenous traction force exerted on ECM. For example, researchers applied external force to the cell by manipulating attached microspheres [stimulating individual or few FAC(s)]

[55,56] or substrates (stimulating an entire cell) [57,58] via ligand-integrin bonds and demonstrated that the magnitude of cell-generated traction force increases with increasing magnitude of applied force, which infers force-dependent stiffening or reinforcement of FAC and stress fibers. Such adjustment of the traction force requires the activation of integrin through an attachment to adhesion ligand, which induces the conformational change of integrin cytoplasmic tail that triggers the intracellular signaling cascade leading to changes in the mechanical strength of FAC and stress fiber. It should be noted that ECM also shows stress-stiffening behavior and, therefore, there may exist a mechanical feedback loop between the cell and ECM which balances the forces they exert [59-62].

So how does the force adjustment lead to the directed migration of the cell adherent on the substrate with a gradient in stiffness? The resistance of ECM against the deformation induced by the actomyosin contraction varies depending on the stiffness of ECM at the sites of focal adhesion and is translated into the tension generated on FAC and stress fibers. Therefore, depending on the ECM stiffness, mechanosensitive proteins constituting FAC and stress fiber experience a different level of tension, which then leads to variations of actomyosin contractility and organization, stiffness of FAC and stress fibers, and eventually the traction force that cell exerts on ECM. Particularly, researchers have found that an integrin of the cell adherent on a stiffer ECM experiences a higher tension, which enhances the lifetime and the stability of the integrin-ligand bond due to its catch bond characteristics [63-66]. In addition, it has been suggested that stretch-activated calcium channels (SACs) located at or near FACs experience a higher tension when the cell adheres on a stiffer ECM, which increases the flux of calcium ion into the cytoplasm [67-70]. Therefore, the ECM stiffness may influence the cytoplasmic level of calcium ion, which is known to affect the contractility and reorganization of actin cytoskeleton. Consequently, when the cell adheres on ECM with a gradient in stiffness, there may be gradients in the stability and the strength of the integrin-ligand bond and the cytoplasmic level of calcium ion, as well as the magnitude of the traction force and the organization of actin bundles within the cell, which may act individually or cooperatively to set the preferential direction of cell migration.

Recently, researchers have suggested that the cell-generated traction force is dynamically fluctuating on a soft ECM and the fluctuating force, which repeatedly and centripetally tugs ECM at the site of focal adhesion, plays a key role in sensing the local ECM stiffness and thus durotaxis [53,71]. Several factors are considered to be contributing to the fluctuation of the traction force, including temporal variations in actomyosin contractility, actin assembly, and engagement between actin filament and integrin via FAC. How does the fluctuating traction force affect

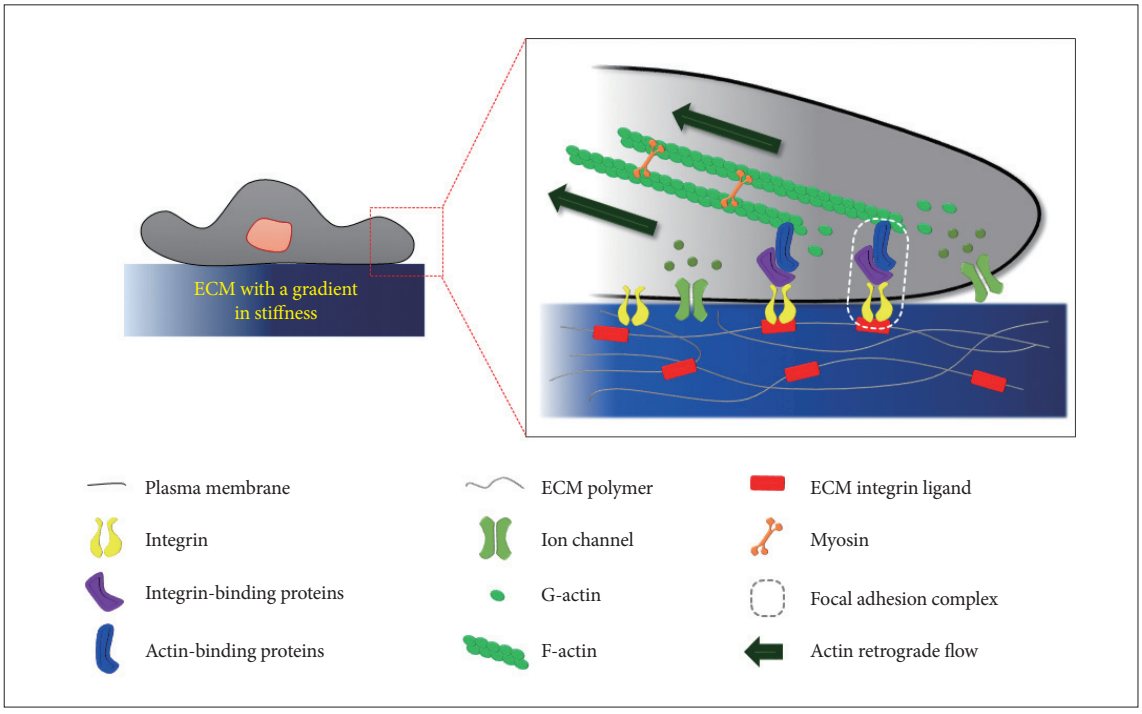

Figure 1. A schematic illustration of molecular organization of FAC and cytoskeletal components in a cell adhered on ECM with a gradient in stiffness. Actin retrograde flow, an indicative of actomyosin contractility, is translated via FAC into the traction force applied to ECM. FAC: focal adhesion complexes, ECM: extracellular matrix.

mechanosensitive proteins such as integrin and SACs in comparison with a sustained or a pulsed traction force then? Researchers have demonstrated that a cyclic application of pulling force, indicative of the fluctuating traction force, on integrin results in two orders-of-magnitude increase in the lifetime of the integrin-ligand bond when compared to a single application of tug on integrin. In addition, it has been demonstrated that activation of SACs, which triggers the transient opening of cell membrane and hence influx of calcium ion, cannot be maintained by the sustained traction force due to channel adaptation; instead, a repeated application of tugging force on SACs is found to be critical for maintaining a high level of cytoplasmic calcium ion via a repeated activation of SACs.

METHODS TO CREATE HYDROGELS WITH THE PATTERNED STIFFNESS

For *in vitro* studies of cell durotaxis, a creation of synthetic ECMs with locally varied stiffness is of utmost importance. Since a number of studies demonstrated that cells cultured on traditional tissue culture polystyrene (TCPS) dishes do not truly reproduce physiological behaviors observed by cells *in vivo* primarily due to large deviations in biochemical and mechanical properties between TCPS dishes and natural ECMs [72,73], efforts have been made to create synthetic ECMs characterized by physiologically relevant biochemical and mechanical properties, including the presence of cell adhesion ligands, porosity, stiffness, and, more recently, spatiotemporal variations of those properties as typically being observed during developments and the progression of pathological conditions [74]. In particular, a material of synthetic ECMs that has been substituting TCPS dishes for *in vitro* cell culture has been mostly a hydrogel, which is a chemically or physically crosslinked polymeric network swollen by water. Therefore, in a number of *in vitro* durotactic studies of cells, strategies have been made to create hydrogels whose stiffness can be spatiotemporally varied.

The stiffness of hydrogel is most frequently measured and indicated by Young's modulus or the shear modulus. The two moduli are linearly proportional to each other provided that the material is isotropic [75], which is valid for most hydrogels in the stress-free state. According to classical theories of network elasticity, the modulus of polymer network is proportional to the density of elastically active polymer segments [76], which is in general an increasing function of densities of polymer network and crosslink [77]. Recent developments in synthetic polymer chemistry and microfabrication techniques have enabled creations of crosslink and hydrogel substrates with spatially and/or temporally controlled densities of polymer network; thus, the stiffness. In the remaining of this section we will discuss general approaches to create hydrogel substrates with spatiotemporal variations in stiffness.

Locally controlling the extent of a photochemical reaction

Strategies to create hydrogel substrates with spatially patterned stiffness can be categorized into three groups as illustrated in Figure 2. One method to pattern the hydrogel stiffness is to spatially control the extent of a photochemical reaction via locally varying a dose of irradiated light. The extent of a photochemical reaction, such as a polymerization initiated by light or a light-induced formation or cleavage of covalent bonds, affects densities of polymer network and crosslink. Therefore, a local variation in the dose of irradiated light results in the patterning of the hydrogel stiffness. Several studies have used a photo-initiator (PI) to create hydrogel, whereupon irradiation of light PIs converts them to active radical species that initiate the chemical reaction; i.e., polymerization to create hydrogel. The dose of irradiate light determines the extent of the conversion of PIs and, therefore, a concentration of active radical species in the precursor solution, which affects the kinetics of radical polymerization [78] and, in practice, the extent of conversion of monomer and crosslinker to the polymer network. As a result, the local densities of polymer network and crosslink, and thus the stiffness of hydrogel, can be manipulated by the local dose of irradiated light.

Moreover, irradiation of light onto polymer chain that contains light-responsive units in either the backbone or the pedant group results in chemical reactions that cleave or connect polymer chains, and thus alter the crosslinking density of the polymer network. Similarly, the local dose of irradiated light determines the extent of photochemical reactions, which affects the local stiffness of hydrogel. Examples of the light-responsive units embedded in the polymer chains include o-nitrobenzyl [38,79-83] or ketal-acetal [84,85] derivatives as photocleavable groups and cinnamic acid, vinyl benzene, benzophenone, or norbornene as photocrosslinkable groups [86-88].

One benefit of using light in patterning the local stiffness is its ability to control the local stiffness in a time-dependent manner while cells are being cultured on the substrate. Although the cultured cells may be exposed to a high-energy light such as ultraviolet and/or cytotoxic chemicals such as PI (and its photo-activated radical species) and monomers, and therefore, undergo phenotype changes or even a programmed cell death, a number of studies have reported that cells are tolerant of, and their phenotypes are not apparently affected by, the limited amount of exposure which is specific to the type of cell and cytotoxic substances. A spatiotemporal patterning of the hydro-

gel stiffness by light in the presence of cultured cells has been mainly obtained by a cleavage of the existing crosslinking sites or a formation of the secondary network, which is often referred to as the interpenetrating polymer network IPN [74]. Although the temporal variation of the local stiffness of ECM *in vivo* is known to occur diversely in our bodies, for example during the embryonic development, the differentiation of stem cells, and the progression of pathological conditions such as atherosclerosis, liver fibrosis, and myocardial scarring, there still is a limited amount of *in vitro* studies that created the synthetic ECM with its local stiffness being varied in a timely manner and that investigated its roles in guiding durotaxis and other behaviors, such as adhesion, spreading, or differentiation, of cultured cells [38,89-92]. Several pioneering works regarding the spatiotemporal patterning of the hydrogel stiffness, though most of them focus on cellular behavior other than durotaxis, have been described in detail by other recent review [74].

A modulation of the local dose of irradiated light has been achieved in general by using a grayscale photomask [25,33,93-96] or a sliding mask [33,97]. A grayscale photomask, similar to a neutral density filter with its optical density being spatially varied, can be readily generated on a transparent plastic film on which an opaque ink is printed at a typical resolution of thousands of dpi and, therefore, has been used to create hydrogels with patterned stiffness in a number of durotactic studies. A gray level of the photomask can be created by the halftone printing; arrays of dots as small as a few micrometers with varying size or spacing are printed on the film to generate grayscale patterns. However, arrays of micron-sized dots discretely printed on the photomask cannot effectively produce the grayscale intensity of irradiated light at a small length scale comparable to that of a single cell; i.e., a few to tens of micrometers, although the intensity of irradiated light spreads near the boundaries of dot patterns and becomes gradient due to the scattering of light. Therefore, the study of cellular responses to the mechanical stimuli at or below the length scale of an individual cell has been primarily limited by the resolution of the grayscale photomask [97]. A sliding mask, an opaque plate that slides above the samples at a given speed, can spatiotemporally block the light irradiating over the samples and, therefore, have been used to generate a continuous gradient in the dose of the irradiated light. Although the shape of the grayscale pattern is typically limited to the one-dimensional monotonic growth or reduction of the gray level, the use of the sliding mask allows

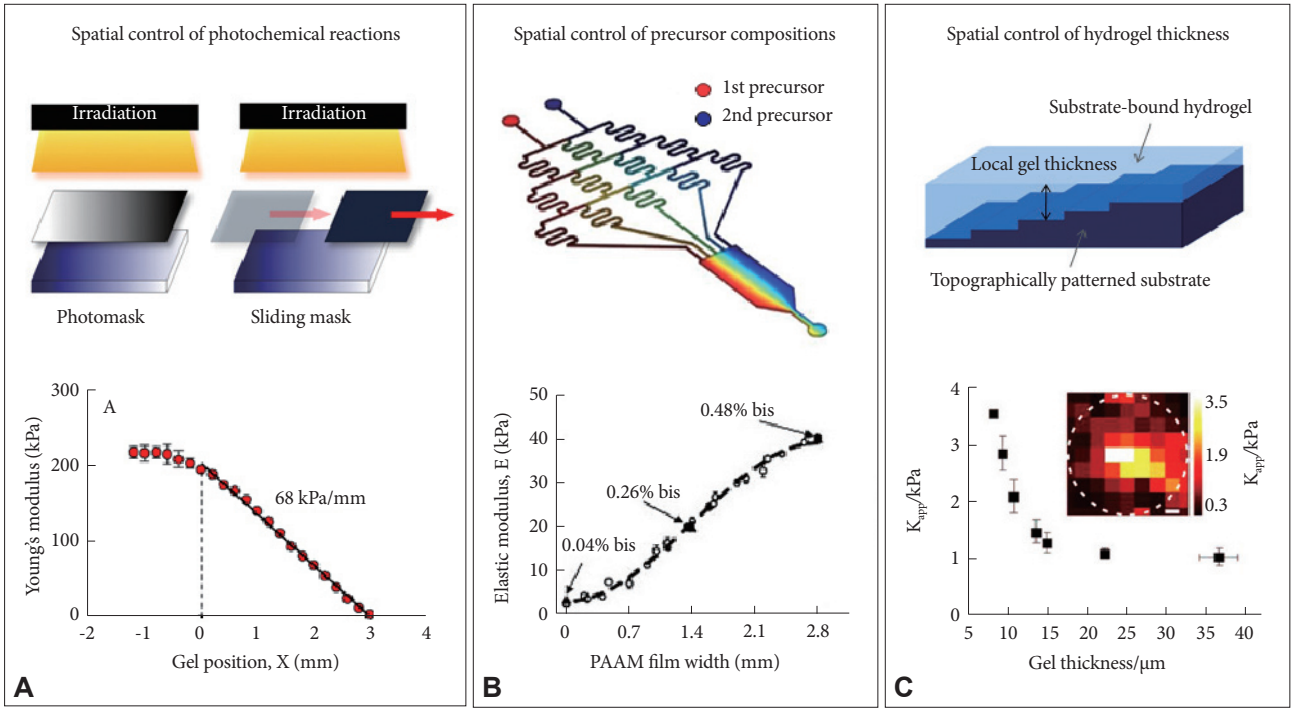

Figure 2. General methods to create hydrogel substrates with spatial patterns of stiffness. (A) A schematic illustration of locally controlling photochemical reactions using either a photomask or a sliding mask and a representative plot of Young's modulus versus a position on the hydrogel (adapted from Sunyer R, et al. PLoS One 2012;7:e46107 [97]). (B) A schematic illustration of a spatial control of precursor compositions using a microfluidic gradient generator (adapted from Giridharan V, et al. J Nanomater 2012;2012 [131]) and a representative plot of elastic modulus versus a width of the hydrogel (adapted from Zaari N, et al. Adv Mater 2004;16:2133-2137 with permission from Wiley [99]). (C) A schematic illustration of locally varying hydrogel thickness using a topographically patterned underlying substrate and a representative plot of apparent stiffness K_{app} versus local hydrogel thickness (adapted from Kuo CH, et al. Adv Mater 2012;24:6059-6064 with permission from Wiley [108]).

creating the stiffness gradient that is continuous below the length scale of an individual cell.

Spatially varying the composition of a precursor solution

Another method to pattern the hydrogel stiffness is to have a spatial variation of the composition of the precursor solution, mostly the ratio of a monomer to a crosslinker, by a hydrodynamic control of multiple solutions. Although a simple method to obtain a spatial variation of the composition has been developed by polymerizing droplets of varying compositions that are placed next to each other and thus create a diffusive interface [51,97,98], the resulting stiffness gradient is significantly limited in the precise control of the width and thus the strength of the gradient. A better control over the spatial variation of the composition has been achieved by a hydrodynamic control of multiple precursor solutions using a microfluidic gradient generator [99-101] or a combination of syringe pumps [34,102] or by a used of special devices [103]. For a microfluidic gradient generator, two or more precursor solutions with varying compositions are separately injected into the microfluidic device made of polydimethylsiloxane (i.e., a gradient generator) and experience a repeated process of mix and split. The repeated process generates a series of the individual stream whose composition becomes an intermediate of two adjacent streams, where the number of the individual stream is set by that of the repeated process. The individual streams are combined in parallel in a wide channel of the gradient generator, resulting in a single stream whose composition varies gradually across the flow direction. A small length scale of the "microfluidic" gradient generator, typically hundreds of micrometers or less in its width and height, renders the combined single stream in the channel to be laminar, which is characterized by a negligible convective mixing, such that the compositional variation across the stream is highly conserved. An irradiation of light onto the stream containing PI results in the formation of hydrogel whose composition and stiffness vary along the width of hydrogel. In addition, a combination of multiple syringe pumps that are programmed to flow the precursor solution with varying compositions and controllable speeds by each pump has been developed. Individual streams from each pump are combined in a single mold, similar to the microfluidic gradient generator, and then polymerized to create hydrogel with a gradient in stiffness.

Locally varying the thickness of a chemically homogeneous hydrogel

The third method to create hydrogel with a patterned stiffness is to locally vary the thickness of hydrogel that is otherwise homogeneous, both chemically and mechanically, and is attached on the rigid substrate. When the cell adheres on a matrix, for example a hydrogel, the cell applies the traction force at the cell-matrix interface that results in a shear strain (i.e., a field of displacement that gradually decays along the depth direction). When the matrix is attached on the rigid substrate, however, the magnitude of the displacement field attenuates [104,105] due to the no-slip boundary condition at the matrix-substrate interface, and the amount of attenuation is inversely related to the matrix thickness. The attenuation in the displacement field in response to the traction force, therefore, renders the cell to overestimate the stiffness of the substrate-bound matrix when compared to its free-standing counterpart. Indeed, the measurement of the local stiffness of the substrate-bound hydrogel by an AFM indentation method supports the idea that the stiffness is inversely related to the matrix thickness [106]. Therefore, the substrate-bound hydrogel that is chemically homogeneous can have a patterned stiffness by locally varying the thickness of the hydrogel [23,107,108]. This method is advantageous in the study of durotaxis because the method allows decoupling the stiffness and the chemistry-dependent physicochemical properties, including a pore size affecting the diffusion of soluble molecules, a surface density of adhesive ligand, and an osmotic swelling, which can rarely be realized by the hydrogels with locally varying densities of polymer network and crosslink. However, the method is limited in the estimation of the stiffness, particularly in the vicinity of step variations in thickness where the displacement field becomes highly complicated [104], and therefore the precise control of the local stiffness is not trivial. Furthermore, a creation of topographically patterned rigid substrate used to generate a local thickness variation is laborious and typically requires a microscopic mold-processing or a multiple photolithographic process, with a swelling of hydrogel typically resulting in the topographic variation at the free surface [108].

Several studies have indicated that there is a critical thickness of the substrate-bound hydrogel, only below which the adherent cell "feels" the presence of the underlying rigid substrate [106] and, therefore, effectively changes its behaviors such as adhesion, spreading, motility, and even stem cell fate. The critical thickness has been reported by a number of studies ranging from as small as one to two micrometers (a characteristic size of an individual FA) [104,109] to tens of micrometers [106,110] and up to a hundred micrometers (a lateral dimension of an adherent cell) [105,108]. The discrepancy in the estimated values of the critical thickness seems to originate from variations in the length scale of cellular behaviors that are of interest, as well as the type of cell and mechanical and chemical properties of the hydrogel used in the studies.

DIRECTED MIGRATION OF ADHERENT CELLS ON HYDROGELS WITH PATTERNED STIFFNESS

The term "durotaxis" was first introduced in the study by Lo et al. [19] in 2000, although earlier studies have reported similar observations such as the directed cell migration on substrates that are mechanically perturbed [111-114] or the altered cellular behaviors in response to a variation in the substrate rigidity [55,56,115,116]. In the study, Lo et al. [19] observed and proposed the mechanism for the directed migration of NIH 3T3 fibroblast cells (3T3s) toward the stiffer region of the hydrogel substrate, which was created by polymerizing two precursor droplets of varying compositions placed next to each other. The authors reported the accelerated protrusion and the increased spreading area of the leading edge when the adherent individual cell moves toward the stiffer region of the substrate. Since the pioneering work, various studies have examined the durotactic behavior of different types of cells on the hydrogel substrates with the spatiotemporally patterned stiffness. This section of the review will not cover details of the roles of key proteins and signaling pathways in mechanosensitivity and durotaxis of cells adhering on substrates with varying stiffness, which have been described in a number of recent studies and also briefly in the earlier section of the review. The remaining part of this section will be assigned to summarizing representative *in vitro* studies that examine the migratory behaviors of various cell types on hydrogel containing continuous gradients of, or discrete patterns of, stiffness.

Different types of cells, mostly mesenchymal stem cells (MSCs) or cells of mesenchymal origin, such as fibroblasts and myocytes, have been investigated for their ability to migrate up along the stiffness gradient of underlying substrate by sensing the local stiffness at the site of adhesion. A number of recent developments in hydrogel substrates with the stiffness gradient is listed in Table 1, and several characterization methods used in the study of durotaxis are also described in Figure 3. As MSCs have been known for their trafficking to the sites of wounds and fibrotic scars *in vivo* [23,25,51], which are rich in ECM proteins such as collagen and thus stiffer than surrounding normal tissues, there has been an inquiry whether a durotactic mechanism underlies the directed migration of MSCs. Several studies have reported that MSCs adherent on hydrogel substrates migrate up along the stiffness gradients that are created by a spatial control of the photochemical reaction [25,96], the composition of precursor solutions [51], or the hydrogel thickness [23]. Such directionally biased migration of MSCs is evident for the stiffness gradient of 1 kPa/mm, which is similar to the gradient found in a normal myocardium *in vivo* and is much shallower than the one found in a pathological condition; e.g., a myocardial infarction with 9 kPa/mm [25]. However, it is found that even in the presence of the stiffness gradient the durotactic behavior of MSCs is abolished and the cells become meandering in a random way when myosin-II is pharmaceutically inhibited by a blebbistatin, suggesting the role of myosin-II in sensing the stiffness of local environment [23]. The migration of MSCs has also been investigated in a 3D environment where encapsulated MSCs experience the stiffness gradient inside the collagen matrix [51]. In this study, the role of myosin-II in durotaxis is further analyzed by each isoform, myosin-IIA (MIIA) which is more abundant and persistently unpolarized, and myosin-IIB (MIIB) which is a minor isoform and polarized toward the cell rear on the stiff matrix. The study suggests that durotaxis of MSCs occurs only when two independent variables, the ratio of MIIB to entire myosin-II and the level of MIIA phosphorylation, both lie within a critical range.

Fibroblasts have also been found to migrate up along the stiffness gradient by a number of studies [19,23,71,95,96,100,102,108]. Human foreskin fibroblasts have been observed to spread faster on a stiffer region of the collagen-coated polyacrylamide (PA) hydrogel [102] and migrate toward a stiffer region of the fibrinogen-grafted poly(ethylene glycol) (PEG) hydrogel when initially adhered on boundaries of discrete variations in stiffness [100]. 3T3s have also been examined for their durotactic ability since the initiative study by Lo et al., [19] where the substrate used is less elaborate in terms of controlling and quantifying the stiffness gradient. Hydrogel substrates with more precisely controlled local stiffness have been created in later studies [95,108], which investigate the effects of the gradient strength and the absolute value of the substrate stiffness on durotaxis of 3T3s. While morphological features, i.e., more spreading on stiffer regions [95,97,108], and directionally biased migration toward stiffer regions [95,108] are observed in common, the absolute value of the substrate stiffness is found to be either irrelevant to durotaxis of 3T3s [108] or, when the value is high enough, restraining the directed migration of 3T3s [95] presumably due to a formation of stronger FACs on stiffer regions, which also seems to lower cellular motility as investigated in another study [117]. The observed discrepancy seems to arise from a variation in the stiffness ranges used for the gradient, which differs by an order of magnitude [95,108], although other variables such as the type of adhesion ligand and/or the hydrogel mesh size might affect the result as well. Changes in activities or formations of cytoskeletal components, such as inhibition of actin polymerization, disassembly of microtubules, or blockage of myosin activity, as well as inhibition of cell adhesion by transforming growth factor beta induced, result in indiscriminate coverage of 3T3s independent

Table 1. A list of recent developments in hydrogel substrates with spatial patterns of stiffness

Cell type	Substrate	Stiffness (kPa)	Method of patterning	Adhesion ligand	Discussions on durotaxis	Ref.
Stem cells						
MSCs	PA hydrogel+ Type I collagen	1–40	Mixing by diffusion between precursor solutions with varying compositions	Type I collagen	Roles of MIIA and MIIB in durotaxis	51
	PDMS+ Type I collagen	5–20	Controlling local thickness of collagen gel on topographically patterned PDMS	Type I collagen	Effects of inhibiting myosin II on durotaxis	23
	PEG hydrogel	3.3–8.2	Controlling local degree of thiolene reaction with photomasks (visible light)	RGD peptide	Direction of cell migration	96
	Methacrylated HA hydrogel	6–25 Up to 90	Controlling local degree of polymerization with sliding masks & photomasks (UV)	RGD peptide	No durotactic study (morphology, spreading)	33
	PA hydrogel	1–14	Controlling local degree of polymerization with photomasks (UV)	Type I collagen	Effect of gradient strength on durotaxis	25
ASCs	PA hydrogel	1, 20, 34	Use of a comb-like reconfigurable device	Fibronectin	Absence of durotaxis	103
	PA hydrogel	1, 10, 34	Controlling local thickness of PA hydrogel on topographically patterned hydrogel	Type I collagen	No durotactic study (morphology, spreading)	107
Fibroblasts						
3T3s	PA hydrogel	14–30	Mixing by diffusion between precursor solutions with varying compositions	Type I collagen	Direction of cell migration	19
	PA hydrogel	1–3.5, 10, 30	Controlling local thickness of PA hydrogel on topographically patterned substrate	Poly (D-lysine)	Effects of inhibiting cytoskeletal components & adhesion	108
	PA hydrogel	1–240	Controlling local degree of polymerization with sliding masks (UV)	Fibronectin	No durotactic study (morphology, spreading)	97
	Styrenated gelatin gel	10–80	Controlling local degree of polymerization with photomasks (UV)	Gelatin	Effects of gradient strength and absolute stiffness on durotaxis	95
HFFs	PEG hydrogel	0.7, 8, 21, 35, 50	Controlling local composition of precursor solution using microfluidic device	Fibrinogen	Direction of cell migration	100
	PA hydrogel	20–150	Controlling local composition of precursor solution using multiple syringe pumps	Type I collagen	No durotactic study (morphology, spreading)	102
MEFs	PA hydrogel	N/A	Locally pulling hydrogel with a blunt microneedle	Type I collagen	Requirement of tugging FA traction in mechanosensing	71
	PA hydrogel	15, 28	Mixing by diffusion between precursor solutions with varying compositions	Type I collagen	Involvement of focal adhesion kinase in mechanosensing	98
Myoblasts						
C2C12s	Polyelectrolyte multilayer film	31, 152	Controlling local degree of dimerization reaction with photomask (UV)	Fibronectin	Effects of stiffness patterns at a subcellular length scale	94
SMCs	PA hydrogel	2.5–11	Controlling local degree of polymerization with photomasks (UV)	Type I collagen	Direction of cell migration	93
	PA hydrogel	3–40	Controlling local composition of precursor solution using microfluidic device	Type I collagen	No durotactic study (morphology, spreading)	99

MSCs: mesenchymal stem cells, ASCs: adipose-derived stem cells, 3T3s: NIH 3T3 fibroblast cells, HFFs: human foreskin fibroblasts, MEFs: mouse embryonic fibroblasts, C2C12s: C2C12 myoblasts, SMCs: smooth muscle cells, PA: polyacrylamide, PDMS: polydimethylsiloxane, PEG: polyethylene glycol, HA: hyaluronic acid, RGD: arginyl-glycyl-aspartic acid, UV: ultraviolet, MIIA: myosin-IIA, MIIB: myosin-IIB, FA: focal adhesion

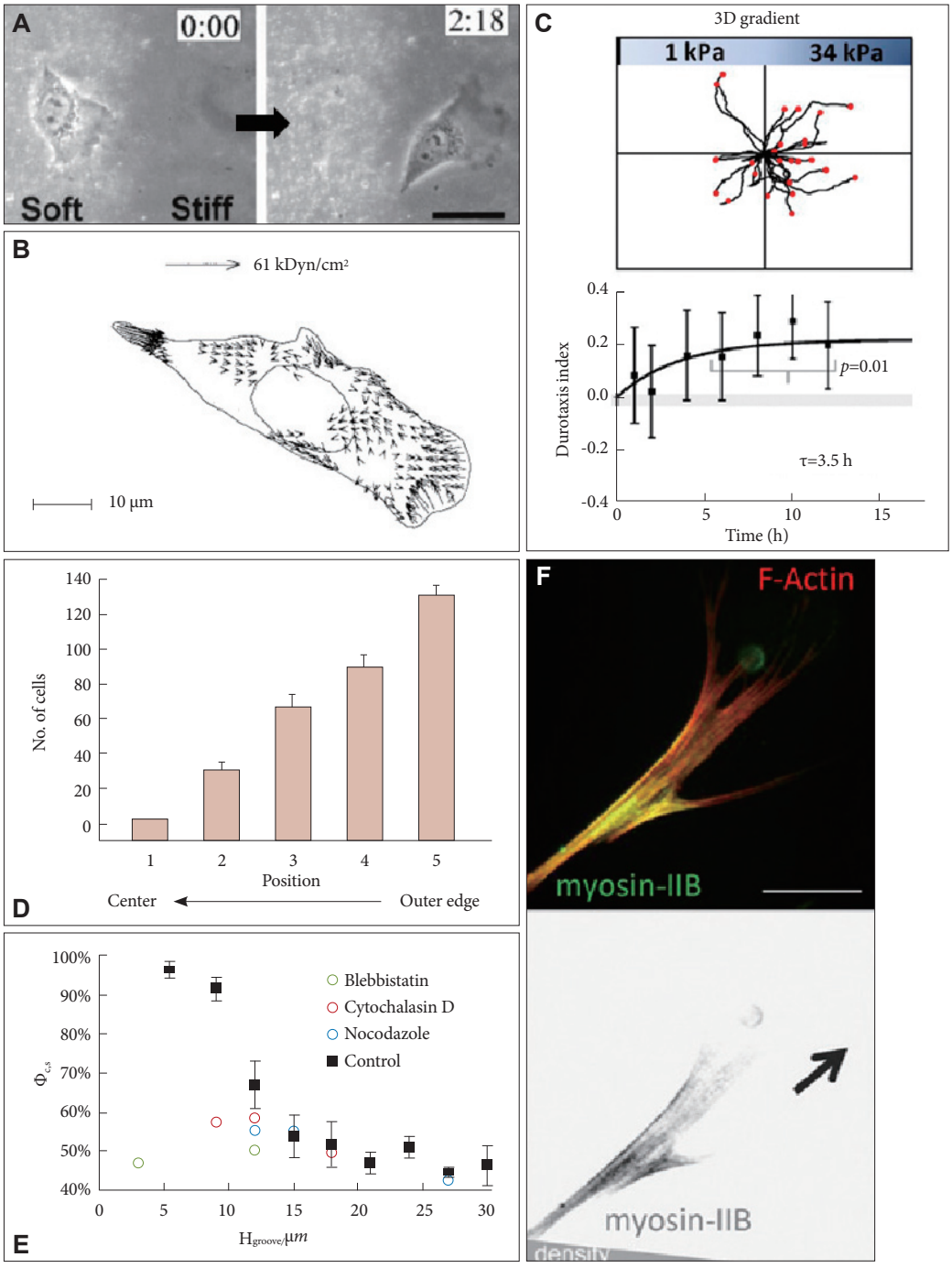

Figure 3. Characterization of cells in the study of durotaxis. (A) An observation by optical microscopy of a 3T3 cell migrating from a soft region to a stiff region. A scale bar is 40 μm (adapted from Lo CM, et al. Biophys J 2000;79:144-152 with permission from Elsevier [19]). (B) A calculation of traction force based on the displacement of fluorescent beads embedded in the hydrogel substrate (adapted from Lo CM, et al. Biophys J 2000;79:144-152 with permission from Elsevier [19]). (C) Trajectories of MSCs encapsulated in the collagen hydrogel with a gradient in stiffness (adapted from Raab M, et al. J Cell Biol 2012;199:669-683 [51]) (top). Durotaxis index averaged for all cells versus time. Details of calculating durotaxis index can be found in [51] (bottom). (D) Histogram showing the number of cells at specific positions on the hydrogel, which becomes stiffer from center to outer edge (adapted from Wong JY, et al. Langmuir 2003;19:1908-1913 with permission from American Chemical Society [93]). (E) A plot showing that an area fraction, Φc,s ,of normal 3T3s increases with decreasing local thickness, which corresponds to increasing apparent stiffness (adapted from Kuo CH, et al. Adv Mater 2012;24:6059-6064 with permission from Wiley [108]). Treatments of 3T3s with inhibitors of cytoskeletal activity or assembly, however, result in indiscriminate coverage of 3T3s irrespective of local thickness. (F) A fluorescence micrograph of MSCs immunostained for MIIB and F-actin (top), and mapping the density of MIIB from the fluorescence micrograph (adapted from Raab M, et al. J Cell Biol 2012;199:669-683 [51]).

of the local stiffness [108], which support previous studies that cytoskeletal activities and receptor-mediated adhesion are essential in locally sensing the environmental stiffness by cells [55,56]. Other types of fibroblasts such as mouse embryonic fibroblasts [71,98] and ligament fibroblasts [23] are found to migrate up along the stiffness gradient.

Other cell types such as vascular smooth muscle cells (VSMCs) [93,99] or cancer cells such as glioblastoma [118] or colorectal adenocarcinoma [108] have been examined for their durotactic abilities. Directed migration of VSMCs from the media, the middle muscular layer of a blood vessel, has been recognized as an essential process that leads to atherosclerosis or restenosis that may occur after a treatment of arterial occlusive diseases with stents or vascular grafts [93]. While directed migration of VSMCs in such pathological or post-treatment conditions might be grounded on existing gradients in concentrations of soluble chemicals or densities of ECM-bound ligands, VSMCs could migrate via durotactic mechanism in the presence of stiffness gradients; e.g., a stiff fibrous cap overlaying a soft necrotic core at the site of atherosclerotic plaques [119] or a metallic stent placed on a soft tissue. The durotactic ability of VSMCs has been examined by culturing the cells on a collagen-coated PA hydrogel with a gradient in stiffness [93], where the cells are found to directionally migrate towards, and accumulate at, the stiffer region. Knocking out focal adhesion kinase (FAK), which is a protein of FAC, results in a random meandering of VSMCs, implying a loss of mechanosensitivity in distinguishing the substrate stiffness.

CURRENT LIMITATIONS AND FUTURE DIRECTIONS OF SYNTHETIC ECM

In general, it is difficult to furnish synthetic hydrogels with characteristics of stiffness possessed by natural ECMs, such as changes in stiffness with time or magnitude of applied strain, which are denoted as stress-relaxation [120] or strain-stiffening [121,122] behaviors, respectively, cell-mediated variations in stiffness by enzymatic degradation of network or deposition of cell-secreted proteins [123-125], or anisotropy of stiffness due to one-dimensional fibrillar structure formed by self-assembly of ECM proteins; for example, type I collagen or fibrin [126,127]. Nevertheless, synthetic hydrogels are advantageous for *in vitro* study of durotaxis over protein hydrogels made of self-assembled ECM proteins in terms of decoupling a gradient in stiffness with that in the density of adhesion ligand [128], each of which is known to induce durotaxis and haptotaxis, respectively, for certain types of cells. For protein hydrogels, changes in stiffness are mostly achieved by altering concentrations of constituting proteins in the precursor solution or the density of covalent crosslinks, which are similar to synthetic hydrogels in the methods of controlling the stiffness. However, a change in concentrations of constituting proteins is accompanied by a change in densities of adhesion ligands, and a formation of crosslinks between adjacent proteins via glutaraldehyde or carbodiimide coupling reactions that consume amine groups in proteins often limit the accessibility of adhesive sequences by cells [128], making it difficult to establish a uniform density of adhesion ligand for stiffness-patterned matrices. For synthetic hydrogels, on the contrary, a spatially homogeneous density of adhesion ligands can be achieved independently of local stiffness, which has been achieved mainly by coating the hydrogel surface with a thin layer of ECM proteins, such as type I collagen or fibronectin, after a completion of patterning stiffness. Although patterning stiffness is generally accompanied by local variations in the network density and the pore size of hydrogel (except for chemically homogeneous hydrogel with a locally varied thickness), the density of ECM coating is found to be uniform and independent of local stiffness, which has been ascertained by a number of studies using a laser scanning confocal microscopy [25,97].

However, there still are a number of issues raised and thus needed to be solved for using synthetic hydrogels in *in vitro* study of cellular durotaxis. First, a thin layer of ECM proteins coated on hydrogel surface for promoting cellular adhesion renders adherent cells to experience the integrated stiffness of both the ECM coating as well as hydrogel. The role of the ECM coating on the integrated stiffness of ECM-coated hydrogel, and thus corresponding responses of adherent cells, has only recently been appreciated by a couple of studies [129,130]. Although the two studies focused on a stem cell differentiation with no considerations in durotaxis and the results are conflicting with each other, one study suggests that stem cells adherent on hydrogels are otherwise identical but varied in the number of anchoring points between ECM proteins and hydrogel and thus the integrated stiffness have undergone differentiation into different lineages [130] whereas the other, which is published later, suggests no such effect [129], so it is highly desirable to examine whether cellular durotaxis is affected by ECM coatings with varying anchoring density.

Second, for synthetic hydrogels, a local variation of stiffness is often accompanied by that of pore size and/or the degree of swelling, which may result in a spatial inhomogeneity of diffusivity, and thus concentration, of soluble chemicals and/or a topographically varied, uneven surface, respectively [128]. Therefore, cells are exposed to gradients in multiple cues; i.e., stiffness, soluble chemicals, and topography, making it hard to isolate durotaxis from other types of directed migration, chemotaxis, and contact guidance.

Third, most of existing synthetic hydrogels used for *in vitro* study of durotaxis lack characteristics of stiffness observed in natural ECMs, such as non-linearity, cell-mediated degradation or deposition of ECM proteins, or microscopic anisotropy as mentioned earlier in this section. Recently, a few studies have introduced synthetic hydrogels characterized by non-linear elasticity [120-122]. Hydrogels made of alginate cross-linked both covalently and ionically show stress-relaxation behaviors where the kinetics of stress-relaxation depends on alginate molecular weight and grafting of PEG chains [120]. Time dependent relaxation of stress generated by cell traction is found to affect spreading and proliferation of 3T3s and differentiation of MSCs. In addition, although there is a lack in cell studies, recent works demonstrate a well-defined, stress-stiffening behavior of synthetic hydrogels made of oligo(ethylene glycol)-substituted polyisocyanopeptides, which form a β-helical structure stabilized by a peptidic hydrogen-bond network [121,122].

In conclusion, although much progress has been made especially with recent developments in synthetic polymer chemistry and microfabrication techniques to create synthetic hydrogels with a spatiotemporal variation in stiffness, there are still a number of concerns regarding the isolation of stiffness with other variables such as the density of substrate-bound adhesion ligand, the concentration of soluble chemicals, topography, and recapitulation of mechanical properties found in natural ECMs such as non-linear elasticity. A proper design of synthetic hydrogel is not only important in providing a better understanding of cellular mechanosensing and durotaxis observed *in vivo* but is also crucial for creating tissue engineering scaffolds.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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