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Engineering Cellular Microenvironments with Photo- and Enzymatically Responsive Hydrogels: Toward Biomimetic 3D Cell Culture Models

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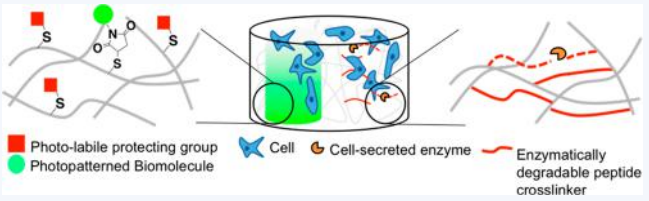
CONSPECTUS: Conventional cell culture techniques using 2D polystyrene or glass have provided great insight into key biochemical mechanisms responsible for cellular events such as cell proliferation, differentiation, and cell−cell interactions. However, the physical and chemical properties of 2D culture *in vitro* are dramatically different than those found in the native cellular microenvironment *in vivo*. Cells grown on 2D substrates differ significantly from those grown *in vivo*, and this explains, in part, why many promising drug candidates discovered through *in vitro* drug screening assays fail when they are translated to *in vivo* animal or human models. To overcome this obstacle, 3D cell culture using biomimetic hydrogels has emerged as an alternative strategy to recapitulate native cell growth *in vitro*.



Hydrogels, which are water-swollen polymers, can be synthetic or naturally derived. Many methods have been developed to control the physical and chemical properties of the hydrogels to match those found in specific tissues. Compared to 2D culture, cells cultured in 3D gels with the appropriate physicochemical cues can behave more like they naturally do *in vivo*. While conventional hydrogels involve modifications to the bulk material to mimic the static aspects of the cellular microenvironment, recent progress has focused on using more dynamic hydrogels, the chemical and physical properties of which can be altered with external stimuli to better mimic the dynamics of the native cellular microenvironment found *in vivo*.

In this Account, we describe our progress in designing stimuli-responsive, optically transparent hydrogels that can be used as biomimetic extracellular matrices (ECMs) to study cell differentiation and migration in the context of modeling the nervous system and cancer. Specifically, we developed photosensitive agarose and hyaluronic acid hydrogels that are activated by single or two-photon irradiation for biomolecule immobilization at specific volumes within the 3D hydrogel. By controlling the spatial location of protein immobilization, we created 3D patterns and protein concentration gradients within these gels. We used the latter to study the effect of VEGF-165 concentration gradients on the interactions between endothelial cells and retinal stem cells. Hyaluronic acid (HA) is particularly compelling as it is naturally found in the ECM of many tissues and the tumor microenvironment. We used Diels−Alder click chemistry and cryogelation to alter the chemical and physical properties of these hydrogels. We also designed HA hydrogels to study the invasion of breast cancer cells. HA gels were chemically cross-linked with matrix metalloproteinase (MMP)-degradable peptides that degrade in the presence of cancer cell-secreted MMPs, thus allowing cells to remodel their local microenvironment and invade into HA/MMP-degradable gels.

INTRODUCTION

Three-dimensional cell culture for more predictive cancer drug screening has emerged over the past decade as an active area of research. Researchers traditionally focused on tissue engineering and regenerative medicine, which involves growing cells in scaffolds *in vitro* for transplantation *in vivo*,[1] are applying their knowledge to tackle the key challenges associated with personalized medicine and drug screening. Such challenges include growing cells *in vitro* to match the proper genotypes, phenotypes, and multicellular architectures naturally found *in vivo*.[2]

In 2004, we demonstrated the first example of nerve cell guidance in 3D hydrogels using photochemically (vs physically) patterned hydrogels, taking what had been achieved in 2D to, literally, another dimension.[3] This work set the stage for an active area of research with the ultimate goal of creating a cell-responsive hydrogel that the cells can remodel as they grow in it, thereby providing a better mimetic of the native extracellular matrix (ECM).[4,5] With the ability to control and manipulate

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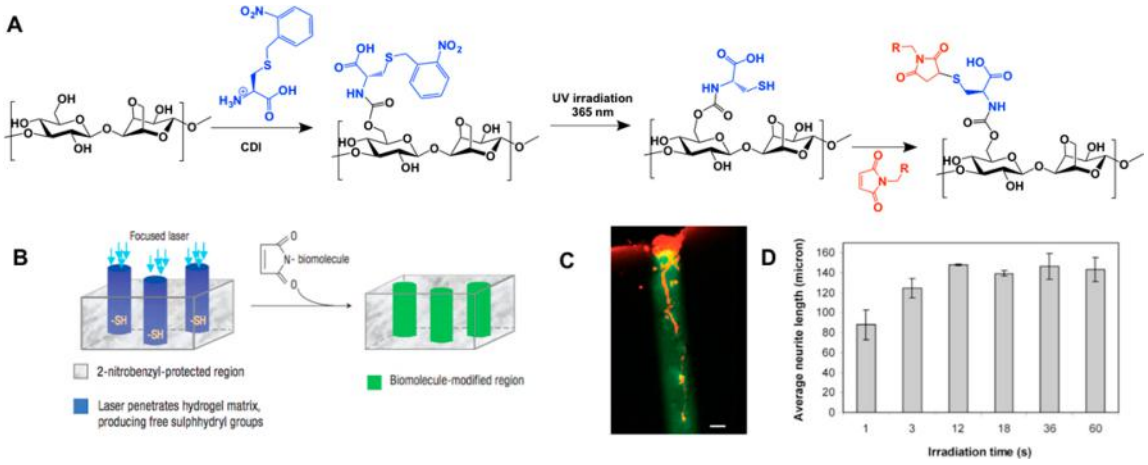

Figure 1. (A) Conjugation of *o*-nitrobenzyl-protected cysteine to agarose. UV irradiation liberates reactive thiols that can react with maleimide-functionalized biomolecules. (B) Schematic of photocleavage within a 3D hydrogel. (C) Image showing immobilized green fluorescently labeled fibronectin-mimetic peptide, GRGDS, in a specific volume and guidance of dorsal root ganglion cells (red) therein. (D) Average neurite cell length is proportional to the time of UV irradiation and therefore peptide immobilization.[3,19]

the ECM *in vitro*, its role in mechanistic pathways can be studied and more efficacious drug therapies can be discovered.

Among the most widely used hydrogel scaffolds is Matrigel, an ECM isolated from Engelbreth-Holm-Swarm mouse sarcoma.[6] While cell culture in Matrigel can support the growth of cellular phenotypes found *in vivo*, such as multicellular breast epithelial cell spheroids,[7] it is limited by batch inconsistencies, undefined components, and insufficient user-specified control of physicochemical properties. In contrast, the use of chemically defined polymers can overcome these limitations. The application of bioorthogonal "click" chemistry, which are high yielding chemical reactions with minimal toxic byproducts, to hydrogels has significantly advanced the ability to tune hydrogel properties to accommodate various cells types for diverse tissue engineering applications.[8] This has allowed for both conjugation of bioactive molecules, and cross-linking reactions to produce bulk hydrogels with uniform and reproducible physicochemical properties. However, while studies using gels that are bulk-conjugated with biochemical ligands[9] and formulated with specific mechanical properties[10] have provided important insights into how cell−matrix interactions dictate cell proliferation and differentiation of various stem cell types, these *in vitro* models are often still overly simplified compared to the native ECM.

In vivo, cell−ECM interactions involve temporally dynamic and spatially defined presentation of physicochemical cues that dictate multicellular events. Cells can migrate along concentration gradients of chemical signals (chemotaxis) and alter the regulation of cellular processes upon binding to biochemical ligands at specific times.[11] Cells can also remodel their surrounding microenvironment, either during normal development or in diseased states, by secreting their own ECM molecules to provide physical scaffolds for cell growth (e.g., laminin, fibronectin, collagen, hyaluronan) or, conversely, by secreting proteinases (e.g., matrix metalloproteinases (MMPs) and cathepsins) that degrade their ECM to allow for cell migration.[12] Thus, to better mimic the dynamic nature of the ECM, hydrogels that can alter their chemical and physical properties in response to external stimuli have been developed. Although beyond the scope of this Account, several excellent reviews have been published describing hydrogels that are responsive to various stimuli (e.g., light, enzymes, pH, temperature, electrical, chemical, and shear stress) for cell culture and tissue engineering applications.[13,14]

We designed *in vitro* 3D cell culture systems that are photo- and enzymatically responsive in order to study cell differentiation and migration in the context of modeling the nervous system and cancer. The hydrogels that we used, first composed of agarose and then of hyaluronan, have evolved in their complexity. Growth factor concentration gradients promote cell guidance, coculture systems provide a means to explore the interactions between multiple cell types, and MMP-degradable cross-links allow cells to remodel their local microenvironment.

PHOTOSENSITIVE HYDROGELS ALLOW FOR SPATIAL CONTROL OF BIOACTIVE MOLECULES

The complex coordination of multicellular events *in vivo* is dependent on how and when physical and chemical cues are presented for cellular recognition. Chemical ligands secreted by neighboring cells (via autocrine, endocrine, or paracrine signaling) can bind to specific cellular receptors, and the defined location of chemoattractants and chemorepellants dictates how cells migrate.[15] Our efforts in designing biomimetic 3D hydrogels with spatially defined presentation of biochemical ligands have focused on using photosensitive hydrogels that are activated for biomolecule conjugation by photoirradiation.

Photolabile protecting groups were first developed in the 1960s, with *o*-nitrobenyzl (ONB) derivatives, which can be cleaved by UV light (at 365 nm), being among the most popular.[16] Several groups, including our own, have conjugated these photoprotecting groups to hydrogels for different applications.[3,17−19] In our early efforts to create spatially defined protein patterns in hydrogels, we used ONB photochemistry to selectively activate functional groups for thiol−maleimide-mediated biomolecule conjugation.[19] Agarose was modified with ONB-protected thiols using carbodiimide (CDI) chemistry, where the primary amine of ONB-protected cysteine is conjugated to the hydroxyl groups of agarose (Figure 1A). Photocaged agarose gels were irradiated with UV light to liberate free thiols in defined volumes, which are subsequently reacted with fluorescently labeled, maleimide-functionalized GRGDS cell adhesive peptides.[20] Peptides were only

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immobilized in irradiated regions (Figure 1B,C). Interestingly, primary rat dorsal root ganglia (DRG) cells seeded on top of these hydrogels grew into the gels only in volumes modified with the active GRGDS and not the scrambled GRDGS. Our subsequent study showed the positive correlation between irradiation time (and therefore the amount of immobilized peptide) and neurite outgrowth from DRG cells (Figure 1D).[3] While this early work clearly demonstrated our ability to control cell function in specific volumes in a 3D hydrogel, peptide immobilization could only be achieved as a uniform column due to the penetration of UV light throughout the entire *Z*-axis.

In 1990, the development of two-photon ultrafast irradiation enabled imaging through live tissues and improved 3D resolution.[21] Conventional nitrobenzyl-derived photocages were first studied for multiphoton uncaging but were inefficiently cleaved.[22] Roger Tsien and co-workers discovered that coumarin-derived photocages could be efficiently cleaved by two-photon irradiation,[23] with 6-bromo-7-hydroxy coumarin (Bhc) being the most efficient photocaging molecule.

Toward improving the spatial control of immobilized proteins within 3D hydrogels, we pursued two-photon microscopy as the irradiation source. Bhc-protected amines[24] and thiols[25] were conjugated to agarose hydrogels. Upon two-photon irradiation, the Bhc group undergoes photolysis and liberates either reactive amines or thiols, which then react to immobilize bioactive molecules (Figure 2). While we achieved

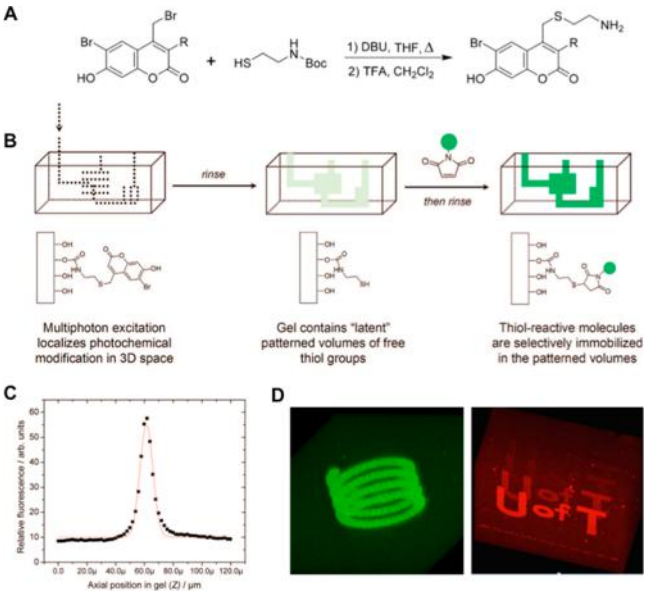

Figure 2. Photopatterning of 3D agarose hydrogels using two-photon irradiation. (A) Synthesis of bromo-hydroxycoumarin-protected thiol. (B) Schematic of multiphoton irradiation in 3D hydrogels to form spatially defined protein patterns. (C) *Z*-axis resolution of photopatterned hydrogels. (D) Photopatterned agarose hydrogels demonstrating defined spatial control.[25]

conjugation of molecules to both amine- and thiol-derived agarose, thiol-modified agarose hydrogels allowed greater selectivity for protein immobilization. Importantly, thiol–maleimide coupling is a clean reaction that preserves the bioactivity of the grafted molecule. Our initial studies demonstrated that Bhc photochemistry can be used to immobilize fluorescent molecules as 3D patterns with distinct spatial orientations within hydrogels (Figure 2C,D). We showed that a biologically relevant protein, maleimide-functionalized vascular-endothelial growth factor-A (VEGF-A, an angiogenic protein), could be immobilized to photolyzed Bhc-thiol–agarose hydrogels and remain bioactive. Immobilized VEGF-A increased the expression of blood phenotypes and induced differentiation of embryonic stem cells cultured therein into blood progenitor cells.[26]

To further develop biomimetic microenvironments for modeling CNS tissue, we sought to immobilize multiple bioactive growth factors in different spatially defined regions within a single hydrogel. In the native tissue, chemical ligands are located in specific regions to facilitate the architecture of multiple cell types. We envisioned a strategy that enables the simultaneous immobilization of multiple growth factors via site-specific protein conjugation, while maintaining their bioactivity with decreased washing time.[27] Instead of directly immobilizing maleimide-functionalized proteins to thiol–agarose hydrogels, as we had done previously, herein we used high affinity binding partners as intermediates (Figure 3). Using agarose modified with Bhc-protected thiols, we sequentially immobilized maleimide–streptavidin[28] (which has high binding affinity to biotin with a dissociation constant, K_d, of 10^{-15} M), and maleimide–barnase[29] (which forms a complex with barstar with a K_d of 10^{-14} M) in defined volumes. Next, we modified recombinant ciliary neurotrophic factor (CNTF) with biotin to yield biotin–CNTF.[30] Similarly, we expressed a fusion of sonic hedgehog (SHH) and barstar, barstar–SHH. Finally, the simultaneous addition of fluorescently labeled biotin–CNTF and barstar–SHH to the previously modified agarose hydrogel resulted in protein immobilization in the desired volumes within the gel (Figure 3B). Importantly, the immobilized proteins retained their bioactivity. Retinal stem cells (RSCs) cultured on SHH-modified agarose hydrogels upregulated Gli2, a transcription factor that is activated by SHH binding to the Ptch1 receptor,[31] to a similar extent as RSCs cultured on agarose hydrogels treated with soluble SHH. Similarly, RSCs cultured on CNTF-modified agarose gels showed an increase in phosphorylated STAT3 expression, which is activated by CNTF binding to the CNTF receptor.[32] This was comparable to RSCs cultured on agarose gels with soluble CNTF and was greater than RSCs cultured on agarose gels in the absence of CNTF.

Having successfully demonstrated that these systems could be used to conjugate multiple bioactive proteins with spatial control, we used this platform to ask important biological questions, such as whether we could guide endothelial cells (ECs) along VEGF-165 (an isoform of VEGF-A)[33] concentration gradients and how ECs influence RSC proliferation and differentiation. Two-photon irradiation was used to create concentration gradients of reactive thiol groups in agarose hydrogels by gradually increasing the exposure to multiphoton irradiation (via the number of scans) and therefore protein conjugation within the depth of the gel (Figure 4). ECs migrated into gels with VEGF-165 concentration gradients (Figure 4B) but neither into gels with uniformly immobilized VEGF-165 nor into gels without immobilized VEGF-165 (Figure 4C). The ECs formed tubular-like structures, mimicking the phenotype observed *in vivo*.

Given that ECs play a significant role in the neural stem cell niche within the brain and that the retinal stem cell niche is highly vascularized, we wondered whether we could gain some insight into the relationship of ECs with retinal stem cells

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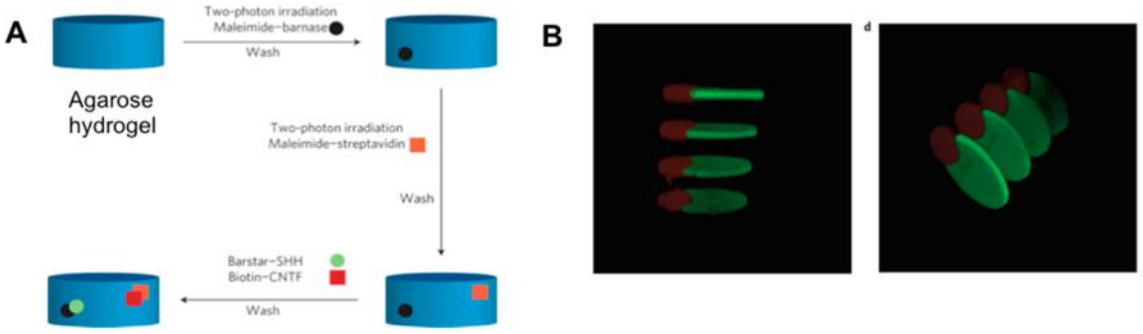

Figure 3. Photopatterning of multiple bioactive growth factors within 3D agarose hydrogels. (A) Schematic of conjugating multiple proteins to ensure bioactivity is retained. (B) Representative images showing conjugation of multiple proteins (red, biotin–CNTF; green, barstar–SHH).[27]

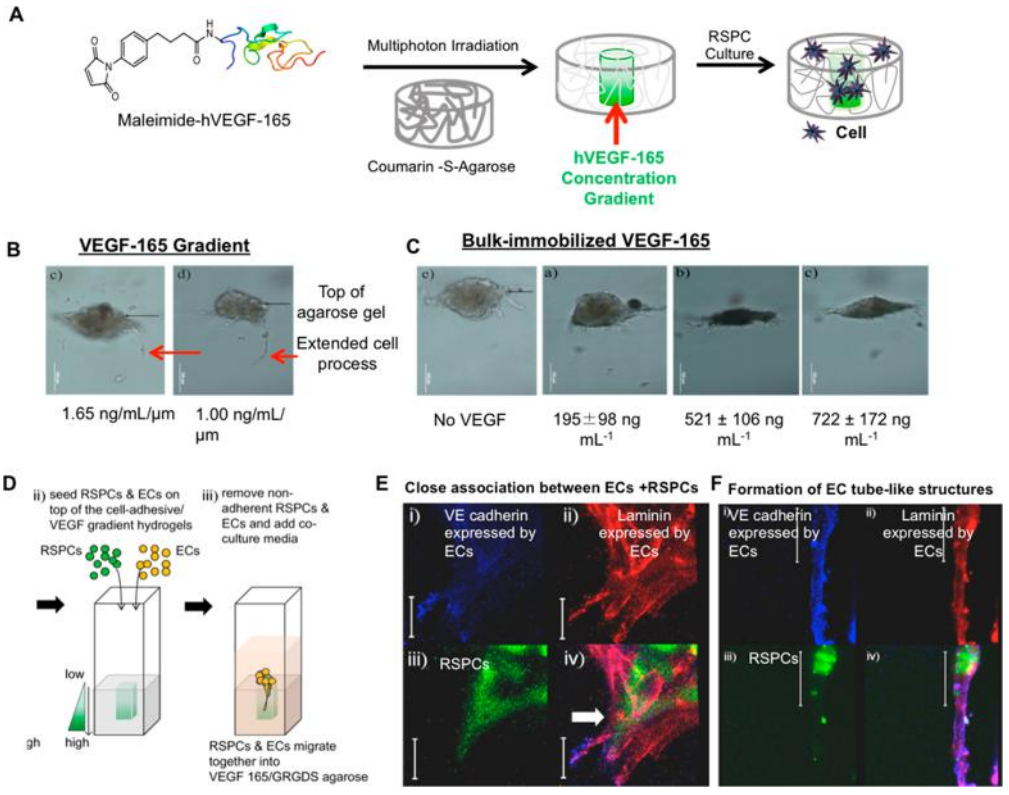

Figure 4. Generation of VEGF-165 concentration gradients in 3D agarose hydrogels enables the study of cellular response to chemotaxis and cell–cell coculture systems. (A) Schematic representation of maleimide–VEGF-165 concentration gradients in agarose hydrogels. ECs migrate into hydrogels (B) in the presence of concentration gradients, but (C) neither in the absence of VEGF nor in the presence of uniform bulk VEGF concentrations. (D) Schematic for coculture system of ECs and RSCs in VEGF-165 concentration gradients with uniform GRGDS immobilized. When cultured in VEGF-165 concentration gradients there is (E) close association between ECs and RSCs and (F) formation of EC tubular-like structures that are stabilized up to 14 days in the presence of RSCs. Parts adapted with permission from ref 33. Copyright 2010 Wiley Interscience. Parts adapted with permission from ref 34. Copyright 2012 Elsevier. Panels B and C adapted with permission from ref 33. Copyright 2010 Wiley Interscience. Panels D and E adapted from ref 34. Copyright 2012 Elsevier.

(RSCs) in our biomimetic hydrogels. RSCs, found in the ciliary body of the eye, are quiescent *in vivo* and, unlike stem cells in the brain, do not proliferate or differentiate. To study these cellular interactions, hydrogels were modified with GRGDS and concentration gradients of VEGF-165, and ECs and RSCs were then cocultured thereon (Figure 4D).[34] ECs and RSCs migrated together into the gels, with the ECs forming stable tubular-like structures for at least 14 d. In the absence of ECs, RSCs did not migrate, and in the absence of RSCs, ECs retracted their cellular processes after 5 days, reflecting a symbiotic relationship between these two cell types (Figure 4E,F). ECs seemed to regulate RSC proliferation and differentiation: ECs maintained RSCs in an undifferentiated state and inhibited proliferation when the cells were cultured with each other. These data show the importance of both cell–cell (EC-RSC) and cell–ECM (EC with VEGF-165) interactions on controlling cell fate.

To better mimic the native tissue microenvironment while maintaining a transparent hydrogel for 3D photochemical patterning, we transitioned to using hyaluronan (HA), a polysaccharide commonly found in the ECM of many tissues, which is overexpressed in breast cancer tissue. It provides a physical scaffold to support cellular growth when cross-linked and acts as a chemical ligand for cell-surface receptors via CD44

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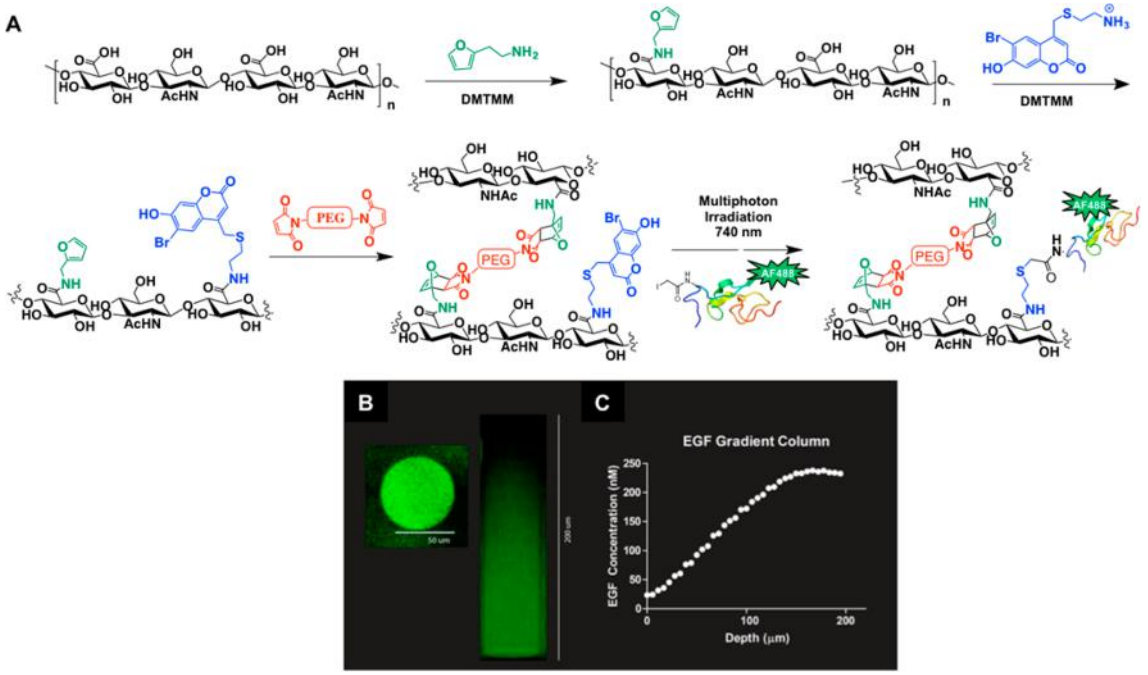

Figure 5. Photopatterning of EGF within 3D hyaluronan-based hydrogels. (A) Synthetic scheme to conjugate Bhc-protected thiols and iodoacetamide-functionalized EGF proteins to HA hydrogels. (B) Representative image and (C) quantification of photopatterned, immobilized EGF concentration gradient along the *Z*-axis. Panels B and C reproduced from ref 38. Copyright 2013 American Chemical Society.

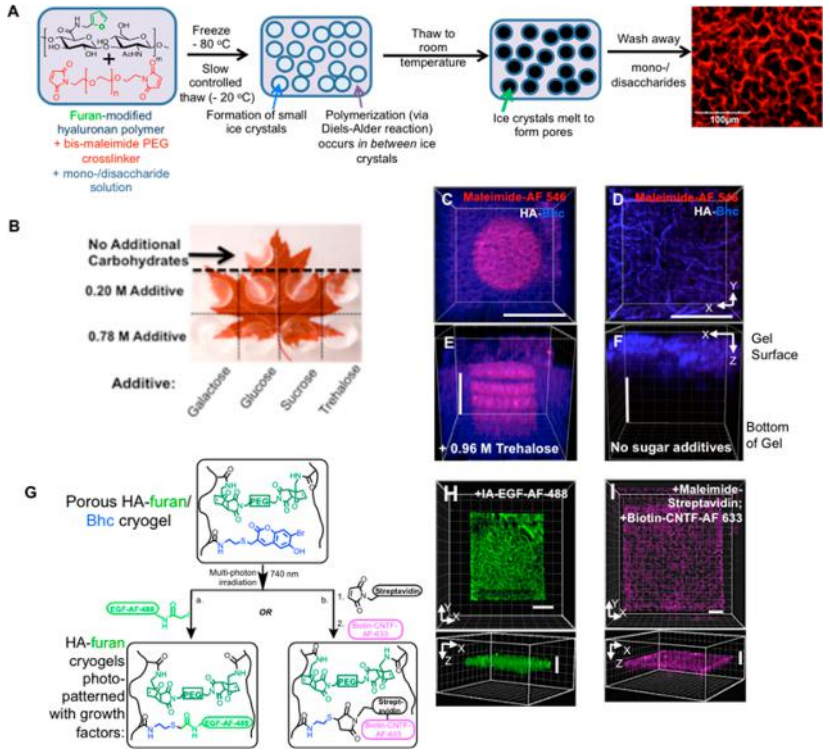

Figure 6. Photopatterning of EGF and CNTF within transparent, macroporous 3D hyaluronan (HA) hydrogels. (A) Schematic representation to prepare HA cryogels via cryogelation. (B) Image showing effects of carbohydrate additives on cryogel transparency. (C–F) Photopatterning is achieved using transparent HA cryogels (red circular discs) with spatial-control and is observed throughout the gel (C top and E side view). No patterning is observed using turbid HA cryogels (D top and F side view), and imaging is not possible below the gel surface. In panels C–F, scale bars = 200 μm. (G) Synthetic scheme for protein conjugation. Confocal images of cryogels photopatterned with (H) IA–EGF–AlexaFluor 488 and (I) Mal-streptavidin/biotin–CNTF–AlexaFluor 633. In panels H and I, scale bars = 100 μm. Adapted from ref 46. Copyright 2016 American Chemical Society.

and RHAMM.[35] While other groups have used different "click" chemistries to form covalently cross-linked HA hydrogels,[36] we focused on forming cross-links using aqueous Diels–Alder click chemistry between furan (as the diene) and maleimide groups

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(as the dienophile), taking advantage of the water-based chemistry and facile nature of these reactions.[37−39] HA was modified with furan via amidation chemistry between furfurylamine and the carboxylic acid of the glucuronic acid moiety of HA, with DMTMM as the coupling reagent (Figure 5A). To enable photopatterning, Bhc-protected thiols were similarly conjugated to the HA backbone. Reaction of these modified HA polymers with bis(maleimide)−poly(ethylene glycol) cross-linkers resulted in stable hydrogels with tunable mechanical properties. Using two-photon irradiation, concentration gradients were formed using a modified growth factor, iodoacetamide−epidermal growth factor (IA-EGF, Figure 5B,C), which is upregulated in many tumor microenvironments.[38]

The topography and porosity of the native ECM are important physical cues that dictate cell growth, differentiation, and migration.[40] While macroporous hydrogels have been prepared using many techniques,[41] their preparation generally results in opaque hydrogels that renders them difficult for imaging using conventional microscopy techniques.[42] For the preparation of macroporous hydrogels using the cryogelation technique, strategies to improve their optical transparency include using rationally designed amphiphilic polymers[43] or additives such as sodium nitrite, DMSO, or glycerol.[44,45] While successful for synthetic and protein-based polymers, these strategies were unsuccessful for polysaccharide-based polymers. Recently, we reported that mono- or disaccharide additives improve the transparency of macroporous polysaccharide hydrogels (including hyaluronan and methyl cellulose) (Figure 6A,B).[46] By improvement of the optical transparency of these cryogels, cells cultured therein are readily visualized using conventional microscopy, and importantly, these cryogels can be photopatterned (Figure 6D,F). In contrast, conventional macroporous cryogels, which are opaque, cannot be photopatterned in 3D (Figure 6C,E), even though multiphoton irradiation is normally able to penetrate through live tissues. We successfully used multiple conjugation strategies to photopattern either maleimide−streptavidin/biotin−CNTF or iodoacetamide−EGF into these transparent, macroporous hydrogels (Figure 6G−I).

Despite successful photopatterning of Bhc-protected agarose and HA gels, the efficiency of Bhc photocleavage is relatively low. DiStefano and co-workers recently discovered that Bhc conjugated to thiols can produce an undesired byproduct that quenches the formed thiols, resulting in decreased conjugation of the thiophilic binding partner (e.g., maleimides, acrylates), Figure 7A.[47,48] Incorporation of a methyl group to Bhc (i.e., 3-methyl-6-bromo-7-hydroxy coumarin, mBhc) overcomes this issue, as the exocyclic methyl group of mBhc forms a less stable adduct and prevents the undesired 1,3-sulfide shift (Figure 7B,C). By chemically conjugating thiol-protected mBhc to our HA polymers, we achieved improved chemical immobilization following two-photon irradiation, as evident by increased sensitivity and lower background compared to our previous system using Bhc (Figure 7D,E).[48]

For truly biomimetic *in vitro* models, both spatial and temporal control of biomolecules is important. For temporal control, photocaging groups have been used to mask the bioactivity of proteins and peptides until they are activated by photo-irradiation.[47,49,50] In addition, the physical properties of hydrogels have been manipulated photochemically in the presence of live cells. By incorporation of photodegradable cross-linkers in PEG-based hydrogels, irradiation resulted in

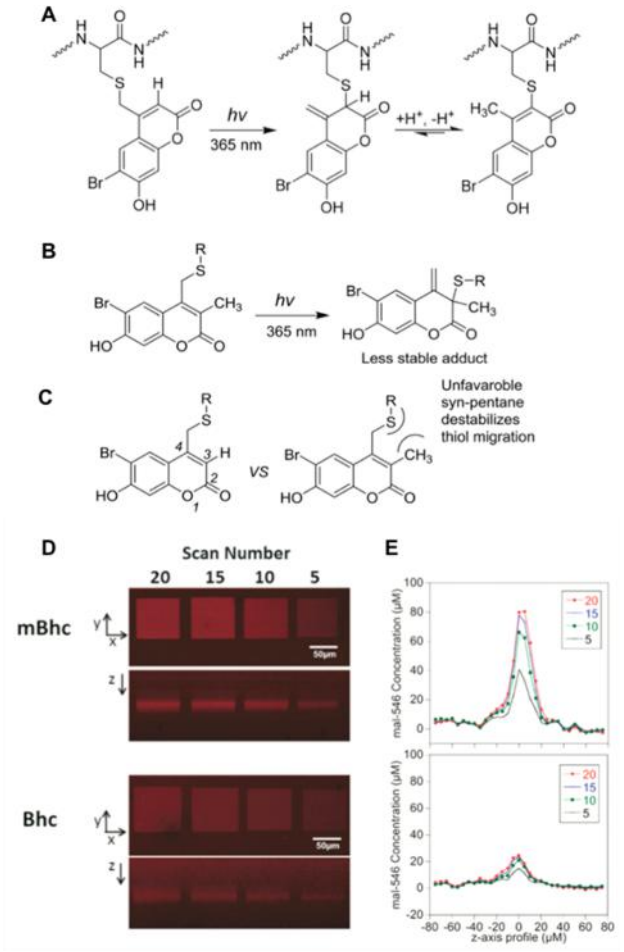

Figure 7. Improving two-photon photopatterning using a novel coumarin photocage. (A) Photoinduced rearrangement of cysteine-bromo, hydroxy-coumarin (Bhc). Methyl-Bhc (mBhc) prevents 1,3-sulfide shift upon photoirradiation as (B) less stable adduct and (C) *unfavorable* conformations for thiol migration are formed. (D) Confocal images and (E) quantification of immobilized maleimide−AlexaFluor 546 fluorescence in HA−mBhc and HA−Bhc hydrogels following multiphoton irradiation at 740 nm with increasing numbers of scans (i.e., increased multiphoton irradiation). Adapted from ref 48. Copyright 2016 Royal Society of Chemistry.

local degradation of the hydrogels to yield softened regions[17,18] or to allow retrieval of specific cells for subsequent studies.[51] Conversely, photochemistry can also be used to stiffen hydrogels, affecting the differentiation of mesenchymal stem cells.[52,53]

CELL-MEDIATED DEGRADATION OF CELLULAR MICROENVIRONMENTS

Cell migration plays an important role in a variety of biological phenomena including tissue morphogenesis and cancer metastasis. Intrinsic cellular properties, as well as cues from the microenvironment are involved in cell migration. Cells can undergo morphological changes or secrete proteases (e.g., MMPs) that degrade their surrounding microenvironment to enable their migration.[54] Engineered degradable matrices have revealed important insights into the interplay between matrix degradation, adhesive properties, and mechanics in orchestrating cellular migration. For example, hydrogels comprising poly(ethylene glycol) cross-linked with MMP-degradable

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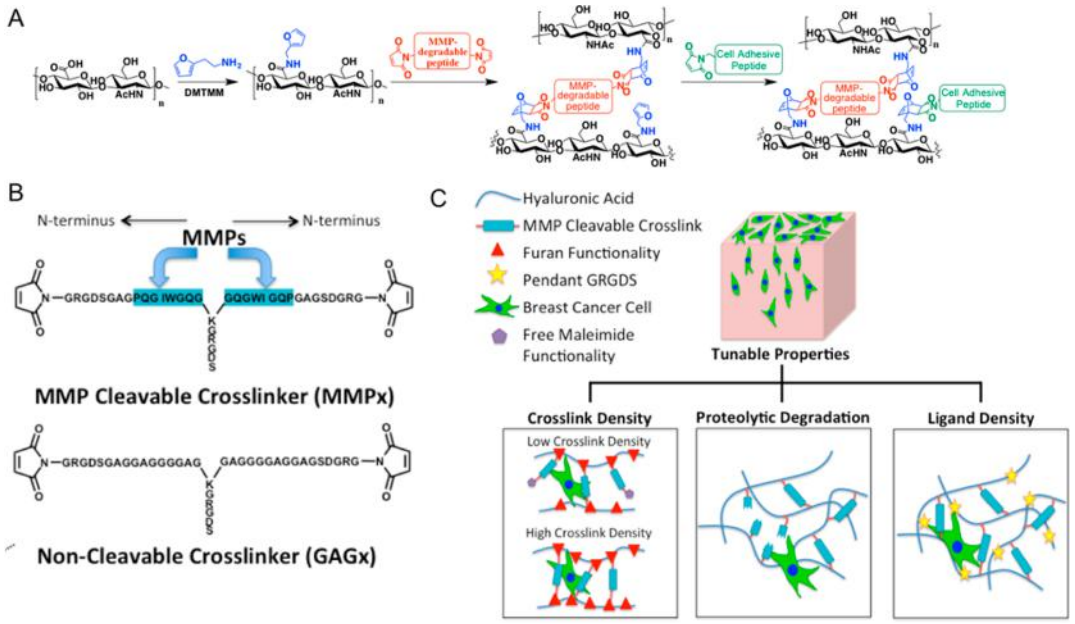

Figure 8. HA/MMP-degradable hydrogels. (A) Synthetic scheme for the synthesis of HA/MMP-degradable hydrogels with pendant cell adhesive peptide immobilization. (B) Bis(maleimide) peptides are designed to be cleavable (or noncleavable for controls) by cell-secreted MMPs. The degradable sequence is highlighted with a blue rectangle. (C) Schematic of HA hydrogels with the different matrix properties that can be tuned. Panels B and C reproduced with permission from ref 57. Copyright 2015 Wiley Interscience.

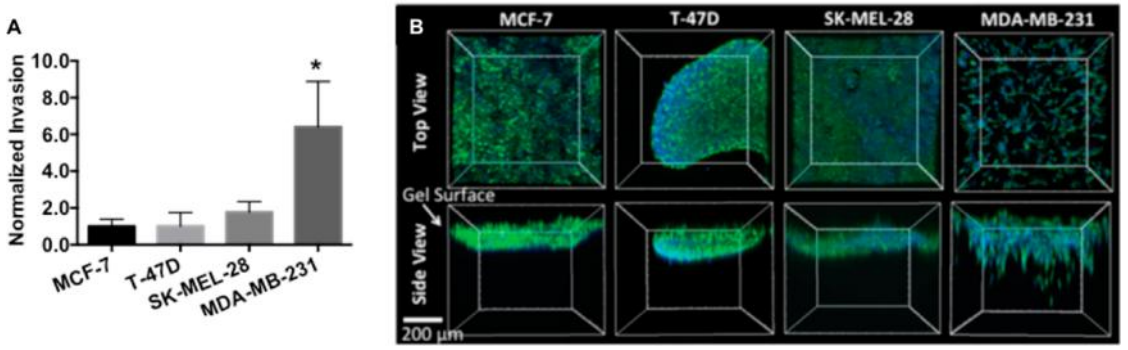

Figure 9. Invasive cell types (MDA-MB-231) preferentially invade into HA/MMP*x*-degradable hydrogels, while noninvasive MCF-7, SK-MEL-28, and T-47D cells remained on the gel surface. (A) Quantification and (B) representative images of cell invasion into HA/MMP*x* hydrogels at 4 d (mean + SD, $n = 3$, $^*p < 0.05$). Blue, DAPI; green, phalloidin. Reproduced with permission from ref 57. Copyright 2015 Wiley Interscience.

peptides (GPQG-IWGQ) have been used to study fibroblast invasion.[55] Cell adhesive peptides that bind to cell-surface integrins are frequently included in hydrogel scaffolds to mediate cell−matrix attachment and regulate MMP expression,[56] and specific peptide concentrations can affect the degree of cell invasion.[55]

Cancer malignancy is characterized by the ability of tumor cells to migrate from the primary site to form secondary tumors. To study the microenvironmental signals implicated in breast cancer cell invasion, our group developed a hydrogel platform with decoupled mechanical and chemical properties to probe their individual contributions to cancer cell invasiveness.[57] As HA accumulation has been correlated with enhanced invasiveness of cancer cells and poor patient outcomes[58] and is an important component of the native extracellular matrix, it is a promising substrate with which to study cancer cell invasion. We used bis(maleimide)-functionalized MMP-degradable peptide cross-linkers to generate HA−furan hydrogels by Diels−Alder chemistry, resulting in stable matrices with tunable mechanical properties that can be degraded by cell-secreted MMPs (Figure 8).[57] Using this platform, cross-link density, proteolytic degradability, and ligand density were tuned independently, such that their individual effects on breast cancer cell invasion could be elucidated (Figure 8C).

Breast cancer epithelial cells seeded on top of these gels were assessed for gel invasion as a function of different microenvironmental conditions. Invasive MDA-MB-231 cells migrated into the gels while noninvasive cell types did not (Figure 9). Cell invasion was affected by the cross-link density of the hydrogel network; densely cross-linked networks inhibited cell invasion whereas softer hydrogels that more closely mimic the mechanical environment of breast tissue promoted cell invasion (Figure 10A−C).[59] The observed invasion was proteolytically driven, as cells invaded into MMP-degradable gels (GPQG-IWGQ) but not into nondegradable gels (GAGGGGAG) (Figure 10D,E). Interestingly, cells did not invade weak gels that were cross-linked with nondegradable peptide cross-linkers, underlining the importance of active MMP degradation

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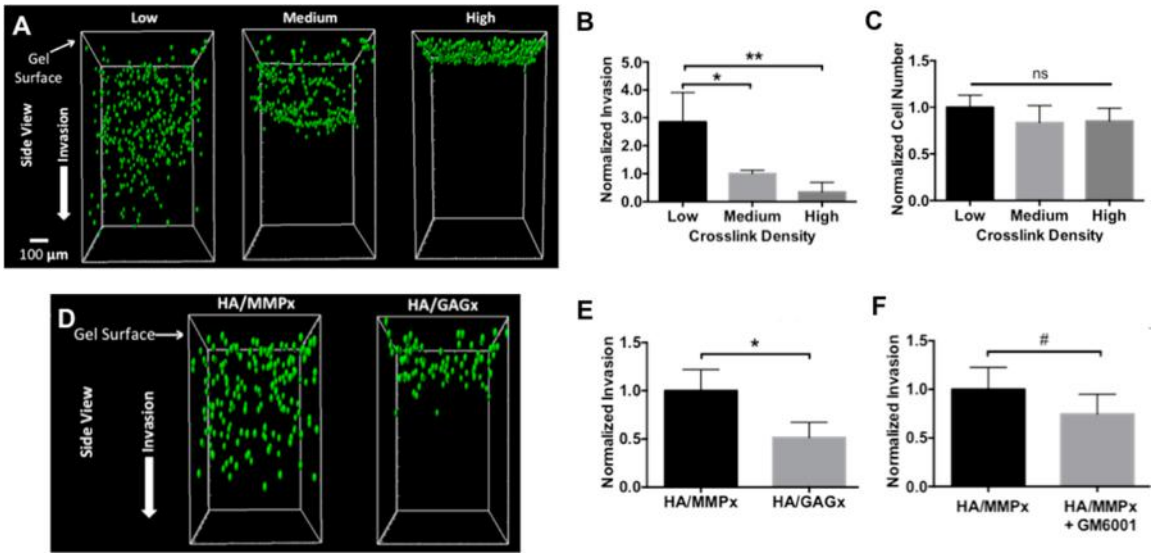

Figure 10. Factors affecting cancer cell invasion. (A) Representative images and (B,C) quantification of MDA-MB-231 cells. (A) Cell nuclei are marked with ovals for visualization. (B) Cell invasion and (C) cell number into HA/MMP*x* hydrogels with varying cross-link densities. (D,E) MDA-MB-231 cell invasion into HA/MMP*x* and HA/GAG*x* (MMP nondegradable cross-linker) hydrogels and (F) HA/MMP*x* hydrogels with GM6001 MMP inhibitor. $n \geq 3$, mean + SD, #$p < 0.10$, *$p < 0.05$, **$p < 0.01$. Adapted with permission from ref 57. Copyright 2015 Wiley Interscience.

by invading cells. Moreover, addition of MMP-inhibitor GM6001 attenuated cell invasion (Figure 10F).

These MMP-degradable hydrogels provide a platform to study breast cancer cell invasion and demonstrate the importance of independently tuning various microenvironmental stimuli in ECM-mimetic hydrogels to understand their role in cancer cell invasion and proliferation. While this study used a single cell type, more complex systems such as coculture with multiple cell types will provide valuable insights into the interplay between cells.[60]

In addition to protease-mediated cell infiltration through the ECM, there are other mechanisms of cancer cell invasion.[61] Cells that lack ECM proteases or are treated with protease inhibitors can switch to protease-independent migration mechanisms, where they exert mechanical forces to reorganize their surrounding matrix while morphologically deforming to migrate through their environment. For example, Aung et al. demonstrated that MDA-MB-231 breast cancer cells migrate into Matrigel networks in a protease-independent manner at low mechanical resistances.[62] Hydrogels cross-linked using dynamic and reversible bonds will allow cells cultured within to deform and remodel their environments through cell-initiated mechanical forces. New cross-linking strategies have recently been developed utilizing reversible chemical bonds and self-assembly of guest–host functional groups to create viscoelastic hydrogels which exhibit stress-relaxation properties.[63,64] In addition, hydrogels containing biochemical ligands that have the ability to "slide" along the polymeric PEG backbone facilitate ligand remodeling by cells cultured therein.[65] The combination of these strategies, along with protease-degradable hydrogels with tunable protease specificity and degradation kinetics,[66,67] will provide a more accurate model toward studying cancer biology, and how dynamic extracellular stimuli can influence cancer cell invasiveness. Importantly, these platforms may provide a better tool with which to perform drug screening to discover drugs that can prevent cancer cell invasion.

SUMMARY AND OUTLOOK

Over the past decade, our lab has developed extracellular matrices (ECMs) with defined chemical and mechanical properties to better mimic the dynamic nature of native ECMs and to bridge the gap between the overly simplistic conventional 2D polystyrene environment and the very complex microenvironment found *in vivo*. We have focused on using polysaccharide-based hydrogels, as these can have inherent biochemical properties unlike synthetic polymers and can be modified to independently tune their chemical and physical properties. By incorporating photo- and enzymatically sensitive molecular motifs, we have prepared hydrogels that are responsive to multiphoton irradiation or cell-secreted enzymes.

Multiphoton irradiation enables the immobilization of bioactive growth factors with 3D spatial control to create chemical concentration gradients and patterns. With an interest in understanding the retinal stem cell niche, we used these gels as tools to study the effect of the surrounding microenvironment on the interactions between retinal stem cells and endothelial cells. We envision that these biomimetic hydrogels, immobilized with spatially controlled growth factors, will enable other important biological questions to be answered, such as factors responsible for chemotaxis, cell differentiation, and self-assembly of higher ordered multicellular architectures. We developed a novel strategy to simultaneously conjugate multiple growth factors using varying binding partners (i.e., biotin/streptavidin and barnase/barstar), thus improving their bioactivity compared to sequential protein conjugation, which can result in protein denaturation. In order to apply multiphoton patterning to macroporous gels, which are usually opaque, we incorporated mono- and disaccharides during cryogelation, thereby being the first to report optically transparent, macroporous polysaccharide-based cryogels. Now conventional confocal microscopy can be used to visualize cells cultured in these 3D, macroporous scaffolds, likely resulting in an expanded use of polysaccharide cryogels for 3D cell culture. Furthermore, while 6-bromo-7-hydroxy coumarin (Bhc) is often used for multiphoton chemistry, we recently reported

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improved efficiency of multiphoton patterning in 3D HA hydrogels using a novel 3-methyl-6-bromo-7-hydroxy coumarin (mBhc) photolabile group that was recently developed by the DiStefano lab;[48] the use of mBhc-based hydrogels provides the advantage of improved biomolecule conjugation with decreased irradiation power and number of raster scans (which may otherwise denature bioactive molecules).

We have developed defined 3D hyaluronan (HA)-based hydrogels that can be selectively degraded by MMP-secreting breast cancer cells to enable their invasion into and remodeling of these gels. By independently tuning the chemical and physical properties of these hydrogels, we studied how each of these factors independently affect cancer cell invasion. These gels are beneficial over commercially available Matrigel and collagen, which are conventionally used for cell invasion assays, as their chemical and physical properties can not be independently tuned. While MMP-degradable hydrogels composed of other biomaterials such as PEG have been reported by various groups to study MMP-mediated cancer cell invasion,[67−69] our use of HA-based gels better recapitulates the tumor microenvironment as HA is often overexpressed in cancer ECMs, and cancer cells often upregulate CD44, the HA receptor. Furthermore, while MMP-degradable HA hydrogels have been reported, their applications have been directed toward tissue regeneration (e.g., bone[70] and cardiac[71]) and stem cell differentiation[52] but not to elucidate the mechanism of invasion of breast cancer cells. We envision that these chemically defined, MMP-degradable hydrogels will be valuable for both understanding the role of the extracellular matrix to cancer cell invasion and drug screening applications to discover more efficacious drug targets.

With the continuing development of novel orthogonal functional groups[8,72] and photocages with different reactivities,[47,48] there is great opportunity to incorporate spatially defined chemical cues, temporal-controlled biomolecule activation, and spatiotemporal control of gel stiffness into a single scaffold. Design strategies that combine these aspects with hydrogels that are also responsive to cellular stimuli such as protease secretion and traction forces will enable the design of more complex, biomimetic systems to better study important biological questions *in vitro*.

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Laura Smith received her Hons. B.Sc. in Chemistry at McGill University. She is currently pursuing a Ph.D. in Chemical Engineering and Applied Chemistry at the University of Toronto under the supervision of Prof. Molly Shoichet. Her research involves the development of a biomimetic hydrogel platform for cancer cell culture and 3D drug screening.

Molly Shoichet holds the Tier 1 Canada Research Chair in Tissue Engineering and is University Professor at the University of Toronto. She has published 230+ papers and given 340+ lectures worldwide. She leads a laboratory of 30 and has graduated 150 scientists. She is actively engaged in translational research and science outreach. Dr. Shoichet was the 2015 L'Oreal-UNESCO For Women in Science Laureate for North America and foreign member of the US National Academy of Engineering and holds the Order of Ontario. Her B.Sc. is from the Massachusetts Institute of Technology and her Ph.D. is from the University of Massachusetts, Amherst, in Polymer Science and Engineering.

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