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Decoupling the effects of stiffness and fiber density on cellular behaviors via an interpenetrating network of gelatin-methacrylate and collagen

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ABSTRACT

The extracellular microenvironment provides critical cues that guide tissue development, homeostasis, and pathology. Deciphering the individual roles of these cues in tissue function necessitates the development of physically tunable culture platforms, but current approaches to create such materials have produced scaffolds that either exhibit a limited mechanical range or are unable to recapitulate the fibrous nature of *in vivo* tissues. Here we report a novel interpenetrating network (IPN) of gelatin-methacrylate (gelMA) and collagen I that enables independent tuning of fiber density and scaffold stiffness across a physiologically-relevant range of shear moduli (2–12 kPa), while maintaining constant extracellular matrix content. This biomaterial system was applied to examine how changes in the physical microenvironment affect cell types associated with the tumor microenvironment. By increasing fiber density while maintaining constant stiffness, we found that MDA-MB-231 breast tumor cells required the presence of fibers to invade the surrounding matrix, while endothelial cells (ECs) did not. Meanwhile, increasing IPN stiffness independently of fiber content yielded decreased invasion and sprouting for both MDA-MB-231 cells and ECs. These results highlight the importance of decoupling features of the microenvironment to uncover their individual effects on cell behavior, in addition to demonstrating that individual cell types within a tissue may be differentially affected by the same changes in physical features. The mechanical range and fibrous nature of this tunable biomaterial platform enable mimicry of a wide variety of tissues, and may yield more precise identification of targets which may be exploited to develop interventions to control tissue function.

1. Introduction

Alterations to extracellular matrix (ECM) stiffness and density occur during tissue aging [1] and disease [2–5] and have the potential to impact cell behavior within the tissue. For example, numerous *in vitro* studies have shown that substrate rigidity can influence the organization and generation of intracellular forces [6], overall cell morphology [7,8], and intracellular signaling [9,10], thereby affecting the differentiation of stem cells [11], migration of a variety of cell types [12–14], and invasiveness of cancer cells [15]. While much of this research has been performed on 2D substrates, most cell types *in vivo* are physically supported by a 3D fibrous ECM, the density and structure of which provide contact guidance cues that are important in cell morphology and invasion [16–18]. However, independently examining the function of fibrous ECM stiffness and density in order to determine their individual roles in cellular processes in 3D is a non-trivial pursuit.

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Reconstituted ECM molecules are often used to create 3D environments for *in vitro* studies due to their ability to mimic the natural bioactivity of physiological environments. Such materials are frequently exploited to study stiffness-dependent effects, as increases in ECM density result in reduced fiber flexibility, leading to an increase in the elastic modulus [19,20]. However, this approach does not allow matrix rigidity to be modulated independently of the concentration of bioactive ECM ligands or ECM density. Additionally, both Matrigel and collagen I form gels primarily via non-covalent interactions [21,22], resulting in mechanically weak structures. As most biological tissues are viscoelastic scaffolds with elastic moduli that vary across tissue types (*e.g.,* 0.1 kPa for brain, 100 kPa for soft cartilage) [23], and pathological conditions such as breast cancer progression can alter the compressive moduli within a single tissue from 0.4 to 10 kPa [24], these current methods are able to replicate only a narrow window of physiologically or pathophysiologically relevant mechanics. Chemical modifications to the ECM, commonly through collagen glycation [25] or crosslinking [26,27], can be used to increase scaffold rigidity, but these techniques yield only slight increases in the achievable range of stiffnesses and often present new complications, such as prolonged incubations, the introduction of new bioactive ligands, and/or alterations to the ECM architecture.

Gelatin-methacrylate (gelMA) has recently emerged as an attractive option for creating engineered ECM-based matrices that possess a wide range of physical properties while maintaining constant gelatin concentration [28]. However, while gelatin is identical in protein composition to collagen, it cannot assemble into triple helices, and thereby does not allow the formation of the higher order fibrillar structures exhibited by collagen *in vivo*. Purely synthetic hydrogels, such as polyethylene glycol diacrylate (PEGDA) scaffolds, can also allow independent manipulation of ligand density and matrix stiffness over a wide range of mechanical properties [29,30], but, similar to gelatin, lack the ability to recapitulate the fibrous networks of *in vivo* tissues. This limitation is problematic because fibers may play important roles in directing cell behavior; for example, fibrillar alignment of collagen in breast tumors has been shown to correlate with a worse patient prognosis [31,32]. The influence of fibrillar structure on cellular outcomes was also highlighted by recent work which described a significant decrease in mouse xenograft tumor growth upon attenuation of fiber formation and crosslinking by inhibition of LOXL2 [33]. Here, we report a novel method for fabricating an interpenetrating network (IPN) hydrogel of gelMA and collagen I which allows for a wide variation of shear modulus while retaining the fibrillar structure of collagen. Furthermore, by varying the ratio of gelMA to collagen, this approach also permits manipulation of matrix fiber density without changing the overall protein content. Using this approach to decouple fiber density and scaffold stiffness, we investigated how these individual physical cues influence cell behavior in the context of the tumor microenvironment. Specifically, we quantified the invasion of MDA-MB-231 breast cancer cells, as changes in tissue stiffness and collagen architecture are prominent features of breast cancer progression. Additionally, as angiogenesis is a key process that supports tumor progression [34], we examined the impact of these cues on endothelial cell sprouting to determine how these responses varied for different cell types that may be present within the same pathophysiology.

2. Materials and methods

2.1. Materials and cell culture

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). MDA-MB-231 human breast cancer cells (ATCC, Manassas, VA) and bovine aortic endothelial cells (BAECS, Cell Applications, San Diego, CA) were used until passage 25 and 9, respectively. Cells were maintained at 37 °C and 5% CO_2 in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% Hyclone fetal bovine serum (Thermo Scientific, Logan, UT), 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine.

2.2. Gelatin methacrylation

GelMA was synthesized using a modification of a previously published procedure [35]. Briefly, type-A porcine skin gelatin was dissolved at 10% w/v in phosphate buffered saline (PBS) at 50 °C. While both type A and type B gelatin can be methacrylated, gelMA synthesized with type A is more common in the field and better characterized. Methacrylic anhydride (MA) was added to the gelatin solution using a peristaltic pump at a rate of 200 μL/min under aggressive stirring. Final MA concentrations of 0.25, 2.5, and 7% v/v were used and will be referred to as 0.25 M, 2.5 M, and 7 M herein. The reaction proceeded for 24 h at 50 °C shielded from light. The solution was spun down at 3000 × g for 5 min to pellet unreacted MA and precipitated protein. The supernatant was dialyzed against PBS using 12–14 kDa MWCO dialysis tubing (Spectrum Labs, Rancho Dominguez, CA) for 2 days at 50 °C, at which point the dialysis solution was switched to ddH_2O for another 3 days at 50 °C. Dialysis buffer was changed daily during dialysis. The gelMA solution was filtered, lyophilized, and stored at −20 °C.

2.3. Hydrogel formation and optimization

To generate hydrogels, gelMA was first resuspended at 20% w/v in PBS and incubated in a 50 °C water bath until dissolved. The 0.25 M, 2.5 M, and 7 M gelMA solutions were blended in varied ratios to tune the gel mechanical properties, with the goal of producing three different gel stiffness conditions. The gelMA solution was then combined with the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; 0.05% w/v final concentration) [36], PBS, and collagen (or equivalent amount of gelatin) in a 37 °C water bath. The prepolymer solution was then incubated at 37 °C for 30 min to polymerize the collagen, at which point the gelMA was photocrosslinked via UV exposure at 365 nm (3.4 mW/cm^2) for 5 min at 37 °C (Fig. 1A). The swelling of the gels was measured as percent increase in wet weight after a 24 h incubation at 37 °C in PBS, and an iterative process was followed to modify the starting prepolymer formulations in order to achieve a final, post-swelling concentration of 5% gelMA across all conditions. Briefly, the initial gelMA concentration was adjusted to accommodate gel volume changes that occurred during swelling, and the final gel formulations used 5.5–7.4% initial gelMA concentrations to yield a final gelMA concentration of 5% across all conditions.

Within each stiffness condition, varied amounts of bovine skin collagen (Fibricol – a 10 mg/mL solution of native bovine type I Collagen, Advanced Biomatrix, San Diego, CA), were added to produce gelMA-collagen gels. Fibricol was added to a solution containing gelMA, PBS, and LAP, and the mixture was briefly vortexed and centrifuged, neutralized with 10X PBS and 0.1 M sodium hydroxide, vortexed and centrifuged again, and then crosslinked as described above. This process was used to generate gels at each stiffness containing a final concentration of 0, 1.5, or 3 mg/mL collagen (Fig. 1B). To maintain constant total protein concentration across all conditions, gels that included 0 or 1.5 mg/mL collagen were supplemented with unmodified gelatin to yield a final amount of 3 mg/mL added protein (i.e., no collagen + 3 mg/mL gelatin; 1.5 mg/mL collagen + 1.5 mg/mL gelatin; or 3 mg/mL collagen + no gelatin). The addition of fibrous collagen increases the stiffness of the resulting hydrogel. To compensate for this, an