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A peptide functionalized poly(ethylene glycol) (PEG) hydrogel for investigating the influence of biochemical and biophysical matrix properties on tumor cell migration

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Abstract

To address the challenges associated with defined control over matrix properties in 3D cell culture systems, we employed a peptide functionalized poly(ethylene glycol) (PEG) hydrogel matrix in which mechanical modulus and adhesive properties were tuned. An HT-1080 human fibrosarcoma cell line was chosen as a model for probing matrix influences on tumor cell migration using the PEG hydrogel platform. HT-1080 speed varied with a complex dependence on both matrix modulus and Cys-Arg-Gly-Asp-Ser (CRGDS) adhesion ligand concentration, with regimes in which motility increased, decreased, or was minimally altered being observed. We further investigated cell motility by forming matrix interfaces that mimic aspects of tissue boundaries that might be encountered during invasion by taking advantage of the spatial control of the thiol-ene photochemistry to form patterned regions of low and high cross-linking densities. HT-1080s in 100 Pa regions of patterned PEG hydrogels tended to reverse direction or aggregate at the interface when they encountered a 360 Pa boundary. In contrast, HT-1080s were apparently unimpeded when migrating from the stiff to the soft regions of PEG peptide hydrogels, which may indicate that cells are capable of “reverse durotaxis” within at least some matrix regimes. Taken together, our results identified matrix regimes in which HT-1080 motility was both positively and negatively influenced by cell adhesion or matrix modulus.

Introduction

Metastasizing tumor cells migrate away from the primary tumor, undergo lymphatic or haemotogenous spreading, and implant within a tissue distant from the primary tumor^{1–3}. Thus, a migratory tumor cell experiences a spatially varied myriad of microenvironmental cues as cells move between tissue compartments. Tissue density and biochemical

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composition, as well as interfaces between tumors and surrounding tissue, are important factors during tumor progression. Investigating how these properties contribute to invasion is difficult when using traditional 3D platforms such as collagen or Matrigel, which contain endogenous signaling sites, and thus directly couple biomechanical and biochemical properties. Further, naturally-derived culture materials offer less control over material properties, as they suffer from batch-to-batch variability, are often ill-defined⁴, and depend on extraction techniques⁵ or environmental conditions during gel formation⁶. For instance, mechanical properties of collagen vary widely depending on the conditions used to form gels⁶⁻⁸. It has been reported that a maximum storage modulus < 200 Pa is achieved when formed at standard incubation temperature for cell culture (37°C), even when using a higher range of densities possible for commercially available solutions⁶. Therefore, there is growing motivation to develop new strategies to complement studies using naturally-derived culture materials with platforms that provide stricter control over individual extracellular matrix (ECM) properties.

To circumvent limitations associated with naturally derived materials, synthetic bioinert hydrogels have been employed for cellular studies. Synthetic PEG hydrogels have been formed by a variety of methods, including free radical polymerization⁹, Michael type addition¹⁰, and “click chemistries”¹¹⁻¹³. Synthetic hydrogels have also been functionalized to model critical properties of the extracellular matrix. Matrix metalloproteinase (MMP) degradable peptide cross-linkers allow for matrix remodeling and adhesion peptides for cell attachment, making hydrogels suitable for studies with numerous cell types^{10, 13-26}. Photoinitiated polymerization strategies also provide researchers with temporal and spatial control suitable for mechanical and/or chemical patterning within discrete regions of PEG hydrogels^{11-13, 22, 27-29} or *in situ* changes in matrix mechanics³⁰⁻³³, a property that spontaneous polymerization chemistries such as Michael type addition do not possess. Therefore, while naturally derived materials capture complex features of the ECM, synthetic hydrogels provide a complementary tool for investigating a wide range of questions important for regenerative medicine and disease due to more precise control over biochemical and biophysical matrix properties.

We recently developed a 3D culture platform¹³ using a thiol-ene “click” chemistry³⁴ to form peptide functionalized poly(ethylene glycol) hydrogels, which is flexible towards incorporation of any biomolecule containing a thiol group and has been used to investigate a variety of biological questions^{13-19, 21, 35, 36}. Peptide functionalized PEG hydrogels formed through thiol-ene photopolymerizations provide several advantages as a 3D culture platform, including (1) homogeneous network formation, through a step-growth mechanism (2) cytocompatible polymerization conditions, (3) facile biomolecule incorporation, and (4) the ability to spatially control the reaction through standard lithographic processes¹³. The goal of this work was to demonstrate the utility of peptide-functionalized PEG hydrogels for investigating ECM influences on tumor cell migration. We observed that both adhesion and matrix modulus positively and negatively contribute to cell migration, where a required minimum threshold of adhesion is required for cell motility and a maximum matrix density exists in which cells cannot migrate. Finally, when cells encountered a mechanical interface in which regions of varied modulus were photopatterned within the same hydrogel, they

were able to penetrate from the rigid to compliant regions, but not vice versa, suggesting a “reverse durotaxis” mechanism.

Results

We aimed to demonstrate the benefits of synthetic control provided by peptide functionalized poly(ethylene glycol) (PEG) hydrogels to systematically investigate matrix influences on cell function. PEG hydrogels were formed through photoinitiated coupling of thiol groups within cysteine-containing peptides and alkene groups of norbornene-terminated 8-arm poly(ethylene glycol) (PEG) monomers. The hydrogels were formulated to include a matrix metalloproteinase (MMP)-degradable³⁷ cross-linking peptide flanked with cysteines and a pendant RGD (arginine-glycine-aspartic acid)-containing adhesion peptide terminated with a cysteine^{38, 39}. The thiol-ene reaction proceeds through a radical mediated step growth polymerization upon exposure to 365 nm light in the presence of a photoinitiator (Lithium phenyl-2,4,6-trimethylbenzoylphosphine, or LAP) (Fig. 1). Migration is restricted to proteolytic mechanisms when cells are encapsulated in peptide-functionalized PEG hydrogels due to a mesh size on the order of 10s of nm^{13–15}. Therefore, peptide-functionalized PEG hydrogels are well-suited for investigating proteolytic migration modes and provide a homogenous scaffold in which modulus and adhesion can be investigated while minimizing confounding effects due to porosity or fibril structure.

For synthetic PEG hydrogels, storage modulus (G') directly correlates with cross-linking density (ρ_x), which is calculated based on experimental variables of mechanical properties including mass swelling ratio (q) and G' (Fig. 2B). Using an 8-arm (40 kDa) PEG backbone, G' can be manipulated through varying the –ene concentration, while maintaining a constant thiol:ene ratio. Thus, G' can be tuned to a large range of values based on the concentration of functional groups. For example, hydrogels were formed with storage moduli of ~1 – 40 kPa by simply changing the total –ene concentration from 5 – 50 mM (Fig. 2A). Alternatively, G' can be modulated by varying the network cross-linking density while maintaining a constant 8-arm PEG backbone molecule concentration (i.e., constant –ene concentration) and changing the stoichiometric ratio of thiol relative to norbornene groups. Using a 6mM –ene concentration, the amount of MMP sensitive cross-linker was varied to control the matrix modulus. Rheological measurements determined that the stoichiometric ratio of 0.50:1.0 (thiol:ene) resulted in hydrogels with $G' = 60$ Pa. Increasing this ratio to 0.55:1.0 or 0.60:1.0 resulted in a more cross-linked hydrogel with $G' = 120$ Pa and 250 Pa, respectively, a range that is often studied in collagen and which has been reported for soft tissues *in vivo* (Fig. 2B–C)⁴⁰.

A particular advantage of tuning matrix modulus by changing cross-linking density through altering the thiol:ene ratio, while maintaining a constant –ene concentration is that the norbornene arms not utilized in cross-linking can be functionalized with pendant biomolecules at defined concentrations without substantially altering mechanical properties (Fig. 2B). Due to the importance of adhesion for cell migration^{41–44}, PEG hydrogels were functionalized with peptides containing a fibronectin derived adhesion ligand (CRGDS)³⁹. A non-bioactive scrambled peptide (CRDGS) was also incorporated to maintain a constant total pendant peptide concentration (CRGDS + CRDGS), thus limiting non-specific effects

due to the presence of charged chemical functional groups^{45, 46}. Importantly, hydrogels could be tuned for a wide range of adhesion properties by altering CRGDS concentration (0.0–1.9 mM CRGDS, adjusted concentration, see Methods) without substantially altering matrix modulus (Fig. 2C). Therefore, synthetic ECM formed through thiol-ene chemistries provide a 3D cell culture platform that is well suited for investigating matrix influences on cell function by enabling systematic control over critical matrix properties, such as adhesion and modulus, while minimizing other confounding factors.

We validated the use of PEG hydrogels formed via the thiol-ene chemistry by investigating the role for extracellular matrix properties on migration for HT-1080 fibrosarcoma cells (HT-1080s), a widely studied model cell type for investigating tumor cell motility^{5, 14, 15, 22, 35, 47–52}. We compared measures of cell motility and morphology for HT-1080s embedded in either MMP-degradable PEG hydrogels or collagen, which is a common naturally derived material used to investigate HT-1080 migration^{5, 47, 48, 51, 52}. HT-1080s adopted similar morphologies and migrated in collagen (3.5 mg/mL) similarly to PEG hydrogels (e.g., 60 Pa, 620 μ M RGD), with a comparable fraction of migrating cells ($87 \pm 5\%$ in collagen vs. $82 \pm 4\%$ in PEG hydrogels) and cell speed (39 ± 1 μ m/hrs in collagen vs. 35 ± 1 μ m/hr in PEG hydrogels) observed for both materials (Fig. 3). Thus, synthetic PEG hydrogels can be tailored to recapitulate biologically relevant cell function observed in collagen, a common naturally-derived matrix for 3D cell culture.

Next, we investigated the role of adhesion on HT-1080 motility by systematically varying the concentration of a cell adhesion ligand (CRGDS)³⁹ within PEG hydrogels formed at different moduli. HT-1080 motility was dependent on adhesion, as cell migration was minimal for PEG hydrogels in the absence of active adhesion ligand (0 mM RGD), but increased in hydrogels when RGD was incorporated, even at a very low concentration of ~ 75 μ M (13% – 37% cells migrating more than one cell length in 60 – 260 Pa hydrogels). The fraction of migrating HT-1080s and average cell speed further increased for each of the 60–260 Pa conditions when the RGD concentration was increased to intermediate concentrations (~ 75 to ~ 750 μ M; Fig. 4A). However, the trend in motility diverged for HT-1080s in PEG gels of varying moduli for higher RGD concentrations ($\sim 1,500$ – $1,900$ μ M). Specifically, HT-1080s migrated with decreased cell speed for the 120 Pa condition, resulting in a pronounced biphasic relationship between the fraction of migrating cells and cell speed as a function of adhesion (Fig. 4A). In contrast, HT-1080 motility was only weakly biphasic with RGD concentration for the 60 Pa condition, while migration speed continued to increase for the 260 Pa condition (Fig. 4A). Notably, migration was substantially reduced for all concentrations of RGD for hydrogels with higher moduli (540 Pa), with $< 6\%$ cells migrating regardless of modulus (Fig 4B), which demonstrates that there is a limiting matrix density where motility is inhibited within the range of RGD concentrations investigated.

An important advantage of thiol-ene photopolymerization is that hydrogels can be spatially and temporally patterned using standard lithographic methods to confine light exposure (and the subsequent reaction) to specific regions within the hydrogel (Fig. 5)^{11, 13}. We first formed hydrogels without patterning to demonstrate our approach in which off stoichiometric ratios of monomer functional groups (SH:Norbornene) was used, thereby

leaving unreacted pendant norbornene groups throughout the bulk hydrogel for further modification. Soluble MMP cross-linker and initiator were then swelled into the partially cross-linked bulk hydrogel and exposed to light a second time to controllably increase modulus. The resulting gel modulus increased with exposure time, thereby providing precise control over mechanical properties within the range defined by the partially and fully cross-linked thiol-ene hydrogels (temporal control, Fig. 5A).

Patterned regions of higher modulus were also formed in the hydrogel by using a photomask to couple soluble MMP cross-linker with unreacted norbornene groups in pre-defined regions, which was illustrated using a fluorescently tagged cross-linking peptide to form well-defined stripes of varying cross-linking density within the thiol-ene hydrogel (Fig. 5B). We encapsulated primary human dermal fibroblasts (hDF) in thiol-ene hydrogels with alternating stripes of lower and higher modulus (100 Pa (soft)- 360 Pa (stiff); 200 μ m width) to demonstrate the capacity to alter cell function within the same hydrogel. hDFs were more spread in the 100 Pa regions compared to 360 Pa regions, demonstrating that influences of ECM density on biological function can be investigated within the same material by *in situ* photopatterning (Fig. 5C).

To investigate how a locally changing ECM in the tumor microenvironment might affect tumor cell motility, HT-1080s were encapsulated in hydrogels with a 12 mM -ene concentration and a thiol:ene ratio of 0.28:1 (100 Pa; 1,600 μ M RGD). Then, 200 μ m lines of higher modulus (360 Pa; 2,000 μ M RGD) were formed through *in situ* stiffening (final thiol:ene, 0.35:1). As was observed for 560 Pa thiol-ene hydrogels, HT-1080s were less motile in the 360 Pa region, which lends further support for the negative correlation between quantified migration and matrix modulus above a threshold cross-linking density (Fig. 6). Interestingly, cells encapsulated in the soft region were unable to penetrate the stiff regions and instead migrated backward once encountering the interface or aggregated along the interface, while cells starting in the stiff regions were less motile, but were not inhibited from crossing into the soft region (Fig. 7). Taken together, PEG hydrogels can be formed with regions of distinct biochemical and/or biophysical properties, which provides a synthetically controlled technique for investigating cell function at matrix boundaries.

Discussion

The 3D microenvironment plays an important role during tumor migration and invasion^{49, 53–57}, with adhesion and matrix modulus each implicated in invasion^{54, 58–64}. For example, changes in extracellular matrix (ECM) stiffness have been implicated playing a role in promoting tumor cell migration and invasion^{54, 58–64}. However, the role for stiffness in promoting invasion and metastasis is not clear since *in vivo* extracellular matrix changes that are associated with tumor progression include multiple matrix components, while biochemical (e.g., adhesion ligand density) and biomechanical properties (e.g., modulus) are coupled for naturally derived materials such as collagen or Matrigel. Collectively, these results highlight the complexities of biophysical/biochemical influences on tumor cell function and identify a need for culture materials that provide systematic control over matrix properties.

Here, we investigated tumor cell motility using PEG hydrogels formed through “thiol-ene” photopolymerization to couple thiol groups with alkenes (Fig. 1). This step growth mechanism³⁴ provides strict control over polymer structure and minimizes variability common to other naturally derived and synthetic platforms, and are well-suited for 3-dimensional (3D) cell culture^{13–19, 21, 36}. PEG hydrogels based on thiol-ene chemistry can be tuned within a wide range of moduli corresponding to many *in vivo* tissues^{60, 65} by varying monomer concentration or cross-linking density (Fig. 2). The RGD adhesion ligand concentration was changed with minimal influences on the final gel modulus by varying the ratio of the adhesion peptide and scramble sequence (Fig. 2). Finally, we demonstrated that distinct regions of alternating modulus density could be formed to investigate the influence of changing matrix boundaries on tumor cell migration (Figs. 5–7, Supplemental Movies S2– 3). Thus, peptide-functionalized PEG hydrogels provide a tool that is well-suited for deconstructing biochemical and biophysical influences on tumor cell function by enabling systematic investigation of individual matrix properties, in this case cross-linking density and cell adhesion ligand concentration.

During metastasis, tumor cells invade surrounding tissue and must migrate through several barriers to successfully take residence in distant secondary sites^{1–3}. We investigated HT-1080 migration at 3D matrix boundaries by forming regions of alternating modulus within PEG hydrogels. We note that creating regions of alternating modulus does lead to interfacial boundaries which may have complex affects on hydrogel properties such as nutrient diffusion, but do not believe that this is a barrier to cellular motility, especially as hydrogels were equilibrated in wells with a sink of media for 24 hours prior to migration studies. HT-1080s tended to reverse direction rather than migrating from the lower into the higher modulus region of 3D patterned PEG hydrogels. Our results contrast 2D⁶⁴ and 3D⁶⁶ migration models that predict cells will preferentially migrate from compliant to rigid regions of the matrix, a process called “durotaxis”⁶⁴. The term durotaxis refers to cellular migration across mechanical interfaces⁶⁴, although it is often associated with gradients. In 2D culture, migrating cells have been characterized by increased cell speed and decreased persistence, increased spreading, and preferential aggregation on higher modulus compared to lower modulus regions of patterned hydrogels^{67, 68}. In the case of 3D durotaxis, fibroblasts preferentially aggregated in the stiffer region of non-uniformly compressed collagen matrix⁶⁶, but a biochemical gradient cannot be ruled out since endogenous signals (such as adhesion sites) are also enriched with increasing fibril density. Previously, PEG hydrogels with varying moduli were used to demonstrate that HT-1080s invade a 21.5 kPa matrix, while minimal invasion was observed for a 42 kPa formulation, which is qualitatively consistent with our results here²². However, the barrier presented by the higher stiffness boundary for HT-1080s in softer patterned regions was not due to the inability of cells to migrate, for example due to higher cross-linking, as cells were motile within 360 Pa regions of PEG hydrogels. We further demonstrated that there exists a threshold modulus (560 Pa), above which HT-1080 motility is prevented (Fig. 4B), which is consistent with a previous study that demonstrated glioma migration was blocked in collagen-agarose gels when agarose reached a critical density⁶³. Therefore, while durotaxis may be the driving force for 3D motility within certain matrix regimes, our results demonstrate that there may be a limiting upper boundary in matrix density that prevents migration in the direction of

increasing stiffness, but which is below the critical density that prevents migration when cells are directly encapsulated (~560 Pa). This upper range of motility may be due to the inability of cells to proteolytically degrade the surrounding matrix on the time scales measured. Importantly, HT-1080s were apparently unimpeded when migrating from the higher to the lower modulus regions of the matrix, which suggests that some regimes promote “reverse durotaxis”, which has been predicted computationally⁶⁹, but to the best of our knowledge has not been demonstrated experimentally.

Finally, we note that HT-1080s are an aggressive tumorigenic cell type^{70–72}, which may play an important role in our observations here. Transformation alters cell adhesion properties^{73–80}, cytoskeletal structure^{81–86}, and may induce a more deformable phenotype^{87, 88}. Notably, several migration models implicate strengthening of adhesions in stiffer regions as a driving force for durotaxis^{53, 61, 89–91}. However, durotaxis has been investigated primarily for strongly adherent cell types^{64, 66–68}, whereas HT-1080s migrate with minimal adhesion-dependence and do not require leading protrusions to generate traction, but instead expressing many features of cortical-contractility driven motility from the rear and adopting a distinct phenotype compared to primary adherent cell types^{14, 15, 35}. Further, it was recently reported that tumorigenic human osteosarcoma cell line (U2OS) were relatively insensitive to a stiffness gradient⁹², which is consistent with tumor cells having altered capacity for durotaxis. Therefore, while we cannot rule out matrix regimes in which a wider variety of cell types would be capable of migrating from stiff to soft regions, we speculate that aggressive tumorigenic cell types such as HT-1080s may be capable of reverse durotaxis in 3D matrices due to a weakly adherent phenotype and rear driven migration.

Methods

Cell Culture

HT-1080s (ATCC) were cultured in Minimum Essential Medium (AlphaMEM Lonza) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% penicillin/streptomycin (Gibco). All experiments were performed with HT-1080s passaged less than 12 times. Primary neonatal human fibroblasts were a generous gift from Prof. R. Rivkah Isseroff (University of California-Davis, Department of Dermatology)⁹³, were cultured in Dubellco's Modified Eagle's Medium (DMEM, Gibco) supplemented 10% Fetal Bovine Serum (Gibco) and 1% penicillin/streptomycin (Gibco).

8-arm 40k MW PEG norbornene synthesis

8-arm PEG norbornene was synthesized as previously described, except that 8-arm PEG MW 40k was substituted for 4-arm PEG MW 20k¹³. All reagents were purchased from Sigma-Aldrich unless otherwise noted. All equivalents are with respect to PEG terminal hydroxyls. All glassware was flame dried and kept under an argon blanket. 8-arm, hydroxyl terminated PEG MW ~ 40,000 (JenKemUSA), 5 eq. pyridine, 0.5 eq. 4-(Dimethylamino)pyridine (DMAP, Fluka) were dissolved anhydrous dichloromethane (DCM) to an round bottom flask. In a separate round bottom flask, DCM, 5 eq. N,N'-dicyclohexylcarbodiimide (DCC, Fluka), and 10 eq. 5-norbornene-2-carboxylic acid were

added and allowed to react for 30 mins. Following the reaction, the PEG, DMAP, pyridine solution was added and the mixture was allowed to react overnight, at which point was filtered. The filtrate was discarded and the norbornene functionalized PEG solution was dissolved in chloroform and subsequently first washed with a 0.05 M glycine buffer and then with brine. PEG was precipitated with cold diethyl ether containing 10% hexanes and vacuum dried. Proton NMR confirmed >95% conversion and purity.

Peptide synthesis

Standard Fmoc protected amino acids were purchased from Anaspec. All peptide sequences were synthesized using solid phase Fmoc chemistry on Rink Amide Resin with a Tribute Peptide Synthesizer (Protein Technologies). Fmoc cleavage was performed using 20%/2% pyridine (Aldrich)/1,8-Diazobicyclo[5.4.0]undec-7-ene (DBU, Aldrich) in Dimethylformamide. Amino acid coupling was performed using 1eq (with respect to amino acid) HBTU (Anaspec)/0.04 M N-methylmorpholine in N-Methylpyrrolidone (NMP, Applied Biosystems). Peptides were deprotected and cleaved from the resin using 95%/2.5%/2.5% Trifluoroacetic acid (TFA, Aldrich)/water/Triisopropylsilane (TIPS, Aldrich) at room temperature for 4 hours. Peptides were precipitated into ice-cold diethyl ether and washed 4 times in diethyl ether, pelleted, and dried. A 1.3% linear gradient starting at 5%/95% Acetonitrile (ACN)/water was used to purify the peptides on a reversed phase HPLC C18 column (Waters Delta Prep 4000). Peptide purity was confirmed via matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (Applied Biosystem DE Voyager).

Monomer solution preparation

8-arm 40kDa norbornene functionalized PEG was dissolved to a 10 wt% solution in phosphate buffered saline (PBS, Gibco). The adhesive peptide, CRGDS, and adhesive peptide scramble, CRDGS were added to the desired concentrations (pre-swelling). Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, synthesized as previously described)⁹⁴ was also added and the solution was exposed to 365 nm light to allow for coupling of ligands to PEG. The MMP-degradable peptide KKCGGPQGIWGQCKK was then added to achieve the desired cross-linking density and the solution was diluted with PBS to yield final concentrations of, PEG (3 wt%), LAP (0.05 wt%). The monomer solutions were aliquoted and stored at -80°C.

Rheometry

Rheology on hydrogels swollen in PBS for 24hr at 37°C was performed on an Ares 4400 rheometer (TA Instruments). A parallel plate geometry with a gap of 1mm (10% compression to alleviate slippage) was employed. Strain sweeps were performed to ensure operation in the linear range and a frequency sweep (0.1 – 100 rad/s) at a constant strain of 20% was used for modulus measurements. For hydrogels formed *in situ*, incomplete hydrogels were first formed by reacting monomers in off-stoichiometric conditions (excess norbornene). Hydrogels were then placed in an MMP degradable peptide solution so that the final thiol concentration would be equal to the norbornene concentration. Swollen hydrogels

were tested as above, except that the hydrogels were exposed to 365 nm light after 30 seconds of strain of 20% at a frequency of 10 rad/s.

Mass swelling ratio (q)

Polymerized hydrogels were allowed to equilibrate in water overnight. Hydrogels were patted dry with a Kimwipe and weighed. Hydrogel were then flash frozen in liquid nitrogen and lyophilized overnight to remove water. The dried hydrogels were subsequently weighed and the mass swelling ratio (q) was calculated via the following equation:

$$q = \text{Mass Wet Hydrogel} / \text{Mass Dry Hydrogel}.$$

Equilibrium RGD concentration

While initial concentrations of RGD for the starting monomers were equivalent, differences in swelling between hydrogels of different moduli led to slight changes in the final RGD concentrations (Fig. 2C). Therefore, all RGD concentration values are an adjusted value based on the post-swelling, equilibrium volume, which was determined as follows:

$$q_f = \frac{m_r}{m_d}; q = \frac{m_s}{m_d}; V_s = \left(1 + \frac{(q-1)\rho_p}{\rho_{sol}}\right)^{-1}; V_r = \left(1 + \frac{(q_f-1)\rho_p}{\rho_{sol}}\right)^{-1}; Q_f = \frac{V_r}{V_s}; [RGD]_{equ} = \frac{[RGD]_i}{Q_f}$$

where: q_f = mass swelling ratio after cure; q = mass swelling ratio after equilibrium; m_r = mass of cured gel, m_d = mass of dry gel, m_s = mass of swollen gel, V_s = volume fraction of polymer in swollen gel, V_r = volume fraction of polymer in cured gel, Q_f = volume swelling ratio after cure, ρ_p = PEG density (1.12 g/cm³), ρ_{sol} = sol fraction density (1.0 g/cm³), $[RGD]_i$ = initial RGD concentration, and $[RGD]_{equ}$ = equilibrium RGD concentration.

Cell encapsulation

Cells were trypsinized, suspended, and counted with a hemocytometer. 20k cells were pelleted in a 15 mL conical vial and the supernatant removed. Cells were resuspended in 100 μ L of monomer solution and 30 μ L of this solution was pipetted into a 1 mL syringe with a cut off tip. Polymerization occurred via exposure of the cell suspension to a 365 nm light source at 10 mW/cm² for 2 mins (XX-40 light source, UVP).

Patterning hydrogels

CRGDS, LAP, and norbornene functionalized 8-arm 40 kDa PEG were combined and diluted with PBS (Gibco) to yield final concentrations of 4 mM, 127 μ M, and 20 mM, respectively. This solution was exposed to 365 nm light at 10 mW/cm² to allow pre-coupling of CRGDS to the PEG backbone. The MMP sensitive peptide cross-linker, additional LAP, and PBS was added to this solution to yield final concentrations of 3.36 mM and 127 μ M, respectively. Cells were encapsulated utilizing the procedure outlined above. These PEG hydrogels were then soaked in 200 μ L of a 4.83 mM solution of the MMP degradable cross-linker, LAP (127 μ M) and PBS for one hour in an incubator at 37°C, 5% CO₂. The hydrogels were placed on a quartz photomask containing alternating 200 μ m wide parallel lines. The samples were again exposed to a beam collimated light (365 nm, 10

mW/cm²) to generate regions of higher modulus. Samples were placed in an incubator at 37°C, 5% CO₂ for 24 hours prior to performing migration experiments.

Migration experiments

Migration experiments and image analysis were performed as previously described¹⁵. Time-lapse microscopy was used to track cells in synthetic ECM for 6 hours (15 minute increments) and analyzed using MetaMorph. We note that matrix connectivity, permeability, and elasticity are coupled within PEG hydrogels. Therefore, increased crosslinking density leads to higher G' and decreased permeability, which may influence nutrient exchange within the PEG hydrogel. However, previous studies demonstrated that changes in permeability were minimal within the range described for PEG hydrogels here. Further, HT-1080 migration was investigated after 24 hours of swelling, which was previously shown to be a sufficient time scale for nutrient equilibrium to be reached in PEG hydrogels with cross-linking densities similar to those reported here⁹⁵.

Data analysis

Migration was determined for cells in the middle 500 µm of an ~1000 µm thick PEG hydrogel disk formed as described above. Cells that interacted or divided were not included in analysis. The fraction of migrating cells represents the average of individual hydrogels where a migrating cell was defined as having migrated a distance greater than one cell length (defined as 44 µm)¹⁵ any time during tracking (6 hours). Reported values for experiments in synthetic ECM represent a 3D correction for 2D minimum intensity z-projections used for tracking (multiplied by a factor of $\sqrt{3}/2$, migration was assumed to be isotropic). Experimental values for cell migration speed and distance to origin were determined as an average for all cells (9 total hydrogels: 3 hydrogels/ea. triplicate experiments). All statistical analysis was performed using a two-tailed student's t-test. All error bars represent standard error of the mean (SEM).

3D immunohistochemistry in synthetic ECM

Cells were stained with Alexa Fluor 546 Phalloidin (Life Technologies A22283). A modification of the instructions for focal adhesion staining kit (Millipore, FAK100) was used to allow for diffusion into the synthetic ECM (8-arm PEG hydrogel). All steps were performed at room temperature except antibody incubations (4°C). Specifically, longer incubation times included fixing (30 min), permeabilization (30 min), blocking (1 hr), washing steps (15 min/ea. for all steps, 30 min/ea. after antibody incubations), and antibody incubations (overnight, 4°C).

Conclusions

In this work, we utilized a peptide functionalized PEG hydrogel to examine the influence of matrix adhesion at varying moduli on HT-1080 migration. HT-1080 motility was characterized by a complex trend in which positive and negative matrix effects on motility were observed. Our results add new insight into the role of extracellular matrix properties and their influence on tumor cell migration by identifying trends that have not previously been appreciated, including “reverse durotaxis”, where HT-1080s migrated from rigid to

compliant patterned regions. Notably, tumors have been characterized by substantially higher moduli than both normal tissue and the adjacent ECM⁹¹, which suggests that invading tumor cells may initially be exposed to a reverse stiffness gradient. While further work will be required to identify the extent to which “reverse durotaxis” influences migration and invasion, our results provide new insight into 3D tumor cell motility by taking advantage of synthetic control over biochemical and biophysical matrix properties while minimizing confounding factors. Importantly, synthetic strategies to form patterned regions within 3D matrices offer an important tool for investigating several unresolved questions in tumor progression, such as the effects of tissue interfaces on tumor invasion and motility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Schematic illustrating the formation of peptide functionalized poly(ethylene glycol) (PEG) hydrogels

Peptide functionalized PEG hydrogels were formed via a radical mediated, step-growth photopolymerization ($\lambda=365$ nm, 10 mW/cm²) in the presence of the photoinitiator, LAP (127μ M). An 8-arm, 40 kDa PEG backbone was cross-linked with an MMP sensitive peptide linker (KKCGGPQG↓IWGQGCKK) and functionalized with the pendant adhesive peptide moiety (CRGDS).

Thiol:Ene Ratio	[RGD] (mM)	[RDG] (mM)	G' (Pa)	q	ρ_x (μM) [§]
0.50:1	0	2.4	60 ± 10	54 ± 7	92
	0.01	2.39			
	0.1	2.3			
	1	1.4			
	2.4	0			
0.55:1	0	2.4	120 ± 20	47 ± 4	175
	0.01	2.39			
	0.1	2.3			
	1	1.4			
	2.4	0			
0.60:1	0	2.4	260 ± 30	42 ± 5	365
	0.01	2.39			
	0.1	2.3			
	1	1.4			
	2.4	0			

$$^{\S} \rho_x = \frac{G' q^{1/3}}{RT}$$

Fig. 2. Peptide functionalized PEG hydrogels can be formed with a wide range of mechanical properties

A. The storage modulus (G') can be tuned over a wide range by varying PEG monomer concentrations. **B.** Formulations and mechanical properties including G' , swelling ratio (q), and cross-linking density (ρ_x) of hydrogels employed in study (RGD and RDG reported as pre-swelling concentrations). **C.** In a 6mM -ene solution, the thiol:ene ratio was varied to change the cross-linking density, resulting in a specific storage modulus. For a specific cross-linking density, G' is relatively flat while varying pendent RGD concentrations (RGD

concentrations reported post-swelling to account for actual RGD sensed by cells). (○ 0.50:1, 0.55:1, □ 0.60:1 thiol:ene).

Fig. 3. HT-1080 fibrosarcoma cells (HT-1080s) are characterized by similar migration and morphologies in collagen and PEG hydrogels

HT-1080s adopted similar morphologies (Red = F-actin/Phalloidin, Blue = nuclei/DAPI) in A. collagen (3.5 mg/mL) and B. PEG hydrogel (60 Pa, 620 μM RGD). C. A similar fraction of migrating cells and D. cell speed was observed for HT-1080s in collagen and hydrogels. Quantified migration was determined by analyzing migrating cells. Experimental values for cell migration speed and distance to origin were determined as an average for all cells (9 total hydrogels: 3 hydrogels/ea. triplicate experiments) (n = 90 per condition). Percent

migration values were determined as an average of each of the 9 hydrogels imaged (n=9). All errors reported as SEM.

Fig. 4. Modulus and adhesion both influence cell migration

A. Cells migrate in hydrogels of starting at low RGD concentrations. As adhesion is increased many more cells migrate at intermediate RGD concentrations, a point where migration is insensitive to modulus. At higher concentrations of RGD, modulus plays a dominant role. Cells in compliant (60 Pa) and rigid (260 Pa) hydrogels exhibit high levels of fraction migrating, where intermediate moduli (120 Pa) revert back to lower levels. At low RGD concentrations, minimal speed is observed, while at high levels of RGD, cells migrate faster in rigid matrices (260 Pa) and display a biphasic response in softer matrices (60/120 Pa). □ 60 Pa; ○ 120 Pa; 260 Pa. **B.** Contour plots of fraction of migratory cells, speed, and distance to origin depend on contributions of each modulus and stiffness. Cells become non-migratory at low adhesion levels and high modulus. Cells were tracked for 6 hours, DTO ~ time^{1/2}. Quantified migration was determined by analyzing migrating cells. Experimental values for cell migration speed and distance to origin were determined as an average for all cells (9 total hydrogels: 3 hydrogels/ea. triplicate experiments)(n = 175 per condition; For table of statistical significance, see Supplemental Figure S1.). Percent migration values were determined as an average of each of the 9 hydrogels imaged (n=9). All errors reported as SEM.

Fig. 5. *In situ* stiffening and patterning of hydrogels

Network evolution of hydrogel stiffening. **A.** Incomplete hydrogel networks are originally formed by using off stoichiometric ratio of thiol to -ene. Additional MMP cross-linker is swollen into the hydrogel and polymerized to complete network structure. A range of moduli can be achieved based on the choice of initial and final thiol:ene ratios (starting ratio: ○ 0.20:1, ◆ 0.25:1, □ 0.30:1, ■ 0.40:1, ending ratio 1:1 thiol:ene for all). The solid black line indicates when exposure to UV light was initiated. **B.** A TAMRA labeled MMP cross-linker was used for *in situ* stiffening to visualize patterned regions of stiff hydrogels. The black

regions indicate that no further MMP cross-linker was incorporated. **C.** hDFs spread out in soft regions of the hydrogel but not in the stiff (scale bars = 100 μm).

Fig. 6. Cell migration in patterned hydrogels

A. In patterned hydrogels, HT-1080 cells in 100 Pa regions had approximately **A.** 5 times (52%) the number of migrating cells when compared to 360 Pa regions (11%). **B.** Cell speed and **C.** distance to origin were 25 $\mu\text{m}/\text{hr}$ and 64 μm in the 100 Pa region compared to 10 $\mu\text{m}/\text{hr}$ and 21 μm in 360 Pa regions, respectively. Quantified migration was determined for the entire population of HT-1080s to compare the invasive potential of the whole cell population as a function of properties of the microenvironment. Experimental values for cell migration speed and distance to origin were determined as an average for all cells (9 total hydrogels: 3 hydrogels/ea. triplicate experiments)(n = 104 per condition). Percent migration values were determined as an average of each of the 9 hydrogels imaged (n=9). All errors reported as SEM. (* = $p < 0.01$).

Fig. 7. Cell migration in PEG hydrogels patterned with regions of alternating modulus
Time-lapse video montage demonstrates cells are unable to penetrate the 360 Pa hydrogel region and instead aggregate along the interface in the 100 Pa region.