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Mimicking white matter tract topography using core–shell electrospun nanofibers to examine migration of malignant brain tumors

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Abstract

Glioblastoma multiforme (GBM), one of the deadliest forms of human cancer, is characterized by its high infiltration capacity, partially regulated by the neural extracellular matrix (ECM). A major limitation in developing effective treatments is the lack of *in vitro* models that mimic features of GBM migration highways. Ideally, these models would permit tunable control of mechanics and chemistry to allow the unique role of each of these components to be examined. To address this need, we developed aligned nanofiber biomaterials via core–shell electrospinning that permit systematic study of mechanical and chemical influences on cell adhesion and migration. These models mimic the topography of white matter tracts, a major GBM migration ‘highway’. To independently investigate the influence of chemistry and mechanics on GBM behaviors, nanofiber mechanics were modulated by using different polymers (i.e., gelatin, poly(ethersulfone), poly(dimethylsiloxane)) in the ‘core’ while employing a common poly(ε-caprolactone) (PCL) ‘shell’ to conserve surface chemistry. These materials revealed GBM sensitivity to nanofiber mechanics, with single cell morphology (Feret diameter), migration speed, focal adhesion kinase (FAK) and myosin light chain 2 (MLC2) expression all showing a strong dependence on nanofiber modulus. Similarly, modulating nanofiber chemistry using extracellular matrix molecules (i.e., hyaluronic acid (HA), collagen, and Matrigel) in the ‘shell’ material with a common PCL ‘core’ to conserve mechanical properties revealed GBM sensitivity to HA; specifically, a negative effect on migration. This system, which mimics the topographical features of white matter tracts, should

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allow further examination of the complex interplay of mechanics, chemistry, and topography in regulating brain tumor behaviors.

Keywords

Glioblastoma multiforme; White matter; Nanofiber; Mechanics; Chemistry

1. Introduction

Glioblastoma multiforme (GBM) accounts for nearly 50% of reported malignant brain tumors [1]. GBM is a primary tumor of astrocytes that, despite decades of research, remains resistant to treatment even with advances in surgical techniques, neuroimaging, and adjuvant modalities such as chemotherapy and radiation [2,3]. Median survival for a patient diagnosed with GBM remains dismal (i.e., ~ 1 year) [1] with tumor recurrence and progression inevitable in almost all cases. Recurrent tumors can appear at the site of the original tumor or at seemingly noncontiguous sites [4]. Clinical observations suggest that these tumors migrate as single cells, particularly along white matter tracts [5,6]. For reasons not yet fully understood, migration occurs despite the fact that white matter is an inhibitory substrate for neurite outgrowth and astrocyte migration [7]. It is clear that additional strategies and technologies are needed to understand the complex and unexpected migration behaviors observed in GBM.

Traditionally, cancer cell migration has been assessed using a number of two dimensional (2D) assays, such as the micro liter migration assay [8,9] or the wound healing assay [8]. However, these assays employ rigid plastic substrates that are far from the aligned nanofibrous topography characteristic of white matter tracts. Similarly, the most common three dimensional (3D) migration assays are based on hydrogels, networks of chemically or physically crosslinked natural or synthetic polymers. The most popular natural biomaterial for evaluating tumor cell migration is Matrigel [10–12]. However, Matrigel is a mouse tumor extract that bears little resemblance to the composition of brain, which is substantially different from normal tissues [13]. Specific to GBM migration, several brain mimetic hydrogels, including hyaluronic acid, have been employed by ourselves [14] and others [15–18]. However, although hydrogels are fibrous materials at the micro/nano scale, these fibers are not usually oriented and therefore, do not adequately mimic the aligned topographical features of white matter tracts.

There are few *in vitro* models that attempt to mimic white matter. The most common approaches use oligodendrocytes in primary culture [19] or organ cultures such as brain slices [20] or whole regions of the brain [21]. These are valuable *in vitro* models; however, animal-to-animal variation and the nuances of organ culture can make them challenging to use. In addition, models based solely on organ/primary cultures generally do not permit investigator control of selected parameters (e.g., chemistry, mechanics, or topography) necessary for systematic study.

Polymeric electrospun nanofibers are alternative neural tissue engineering substrates [22–24] that have been used as guides for neural repair and regeneration [22,25,26] and

substrates for Schwann cell maturation [27] and neural stem cell differentiation [28]. Aligned electrospun nanofibers are particularly interesting as neural guides because of their topographical similarity to white matter [29]. Additionally, aligned electrospun nanofibers (i.e., poly(ϵ -caprolactone) (PCL)) reproduce the morphological and molecular signatures of glioma migration *ex vivo* [30,31]. However, to the best of our knowledge, these tunable materials have not been employed previously to examine the role of microenvironment, specifically mechanics and chemistry, on GBM behaviors.

Further, electrospun nanofibers offer a unique advantage as cell culture models: the ability to individually investigate the influence of chemical and mechanical effects on cell behavior. Through the use of core-shell electrospinning, independent materials can be used to form the core and surface of the nanofiber, thus permitting fibers with identical topography, but varying mechanical or surface chemistry features to be constructed. In this study, aligned, core-shell electrospun nanofibers were fabricated using coaxial electrospinning to mimic the topography of the native *in vivo* environment, including white matter tracts. Electrospun nanofibers displayed nearly identical topographical features, whereas mechanical and chemical features were independently tuned to examine their role on tumor cell behaviors. To examine the influence of mechanics, various core materials were used (i.e., gelatin, poly(dimethylsiloxane) (PDMS), and poly(ethersulfone) (PES)) with an identical shell (i.e., poly(ϵ -caprolactone) (PCL)). Similarly, to study the role of chemistry on tumor cell behavior, the glycosaminoglycan hyaluronic acid/hyaluronan (HA), which is found in white matter [32], was spun as a shell on PCL core nanofibers. For comparison, collagen and Matrigel were also used as shell materials on PCL core fibers. These biomaterials are widely applied as 3D cell culture models [33,34]. Using this electrospun nanofiber (ENF) system, the roles of mechanics and chemistry on GBM cell adhesion, morphology, feret diameters, migration speed, focal adhesion kinase (FAK) and myosin light chain 2 (MLC2) expression were examined.

2. Materials and methods

2.1. Preparation of pure PCL and aligned core-shell nanofibers

Aligned core-shell nanofibers were prepared by coaxial electrospinning. For preparation of gelatin core-PCL shell samples, 5 wt% PCL (M_n 70,000–90,000, Sigma-Aldrich, St. Louis, MO) and 6.7 wt% porcine gelatin type A (300 Bloom, Sigma-Aldrich, St. Louis, MO) solutions were prepared separately in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (>99% purity; Oakwood Products, Inc., Columbia, SC) by stirring at room temperature ($\sim 25^\circ\text{C}$) for 24 h. PCL and gelatin solutions were then poured individually into separate 20 cc syringes. One syringe containing the core solution (e.g., 6.7 wt% gelatin) was fitted with a stainless steel 22 gauge blunt tipped needle fed through a hollow, stainless steel T-joint core-shell nozzle (Small Parts Inc., Miramar, FL), whereas the syringe containing the shell solution (i.e., 5 wt% PCL) was connected to nozzle. The syringes were then placed into individual syringe pumps, set to flow rates of 2 mL/h and 4 mL/h for the gelatin and PCL solutions respectively, and electrospun using a DC high voltage power supply (Glassman High Voltage, Inc., High Bridge, NJ) at positive 20 kV, and a 20 cm needle-to-collector distance [35], for ~ 45 min at an average relative humidity of $\sim 30\%$. Using these conditions,

electrospun fiber was deposited on a mandrel coated with tissue culture polystyrene (TCPS) substrates rotating with a linear velocity of 15 m/s to produce aligned core-shell gelatin-PCL scaffolds. Aligned, single material PCL scaffolds were produced using a single syringe fitted with a 20 gauge blunt tipped needle, at a flow rate of 5 mL/h without the T-joint core-shell nozzle and the same spinning conditions stated above except the spinning time was ~ 25 min. For preparation of poly(ethersulfone) (PES)-PCL core-shell nanofibers, 5 wt% PES was dissolved in HFP at 55 °C for the core with 5 wt% PCL used for the shell. After cooling to room temperature, the polymer solutions were placed in 60 cc syringes with a 20-gauge blunt tip needle and coaxially electrospun using a high DC power supply set to a positive 25 kV, a 20 cm tip-to substrate distance, and flow rates of 4 mL/h for both the core and shell for ~ 20 min. For preparation of poly(dimethylsiloxane) (PDMS)-PCL core-shell nanofibers, 1 g PDMS base (Dow Corning Corporation, Midland, MI) was dissolved in a mixture of dichloromethane (DCM) and toluene (weight ratio 3:2) together with 0.1 g curing agent provided by manufacturer. Following this, the PDMS mixture was electrospun as the core with 5 wt% PCL as the shell using a DC power supply set to a positive 25 kV, a 20 cm tip-to substrate distance, and a flow rate of 1 mL/h for the core and 4 mL/h for the shell for ~ 30 min. Samples were then cured for ~ 24 h at room temperature.

Nanofibers with identical cores but different shell surface chemistries were prepared likewise. PCL (5% w/w), 10% w/v acid soluble collagen type I from bovine hide (Kensley Nash; Exton), and 1% w/v Matrigel (BD Matrigel, BD Biosciences) solutions were prepared by stirring with HFP at room temperature (25 °C). A 1.1% w/v hyaluronic acid (HA) (from *Streptococcus equi*, Sigma-Aldrich) solution was prepared by stirring with a 1:1 ratio of deionized water (DI water) and N, N-dimethylformide (DMF) (>99% purity, Oakwood Products, Inc., Columbia, SC) at room temperature. After complete dissolution of the polymers in the solvent, the dissolved PCL, collagen, Matrigel and HA solutions were poured individually into separate 20 cc syringes. Flow rates were set to 4 mL/h for the core (i.e., PCL) and 2 mL/h for the shell (i.e., collagen, HA and Matrigel) solutions, respectively. Aligned electrospun core-shell samples were deposited on a mandrel coated with TCPS substrates rotated with an average linear velocity of 15 m/s, using a DC high voltage power supply, a negative 5 kV was applied to the mandrel and a positive 20 kV was applied to the coaxial nozzle while maintaining the same 20 cm needle-to-collector distance. Core-shell PCL-collagen samples were spun for 30 min, whereas PCL-HA and PCL-Matrigel samples were spun for 35 min. The as-spun ENF mats were placed in a vacuum oven (<30mmHg) at 25 °C for 24 h to ensure removal of residual solvent [36].

As spun-PCL and core-shell electrospun nanofibers were then rendered hydrophilic to support cell culture. [Plasma treatment was not necessary for electrospun nanofibers with varying surface chemistries as only hydrophilic chemistries were used; however, treatment was performed on all electrospun nanofibers to maintain identical conditions]. Nanofibers were air plasma treated by placing samples inside a Harrick plasma cleaner chamber (Harrick Plasma, Ithaca, NY, USA) under vacuum at 1000 mTorr and exposing samples to a plasma radio frequency of 8–12 MHz for 2.5 min. After 2.5 min, samples were removed and kept in a sealed container until use. Following this, ENF mats were cut into ~ 16 mm diameter cylinders using a metal punch (Arch Punch; C.S. Osborne & Co, Harrison, N.J.)

for cell culture experiments. For analysis of signaling pathways via western blotting, mats were cut to press fit into 60 mm petri dishes (Fisher Scientific).

2.2. Morphological, surface, and mechanical characterization of aligned PCL and core-shell nanofibers

2.2.1. Scanning electron microscopy (SEM)—For examination of fiber micro-architecture, ENF discs ($N = 3$) were placed on aluminum stubs using carbon tape (Ted Pella, Inc.), sputter coated with gold for 30 s (Model 3 Sputter Coater 91000, Pelco, Reading, CA) and imaged using a scanning electron microscope (Quanta 200 SEM or XL30F ESEM, FEI Company, Hillsboro, OR).

2.2.2. Fiber diameter, fiber density, and fiber alignment—Individual fiber diameters and fiber density were measured using Image J software (available at <http://rsbweb.nih.gov/ij/>) applied to the SEM images. For measuring fiber diameter, a line tool was used to measure the edge-to-edge distance perpendicular to individual nanofibers ($n = 75$ fibers). For measuring fiber density (also known as line density), a line of known length was drawn perpendicular to the alignment of fibers and the number of fibers crossing the line was manually counted as described elsewhere [26]. For examining fiber alignment, a Fast Fourier Transform (FFT) was used [26,37]. A radial summation of pixel intensities on the FFT output image was performed using the oval profile plug in (Image J) and plotted as normalized intensity or gray value versus degree for each nanofiber examined. The degree with highest intensity was set to $90^\circ/270^\circ$ (as the plot is symmetric) to compare different nanofiber samples.

2.2.3. Mechanical properties of nanofibers using tensile testing—The mechanical properties of core-shell gelatin-PCL, PES-PCL, PDMS-PCL and PCL electrospun nanofibers were determined using a uniaxial bench-top testing machine (TEST RESOURCES- Type R, TestResources Inc, Shakopee, MN). Samples were cut to a gage-length of 20 mm and gage-width of 2.4 mm by placing the fiber mats between two, 2 mm thick stainless steel ‘dog-bone’ shaped templates and carefully cut as described previously [38]. A stainless-steel surgical blade was employed to make the straight cuts of the template, and a 6-mm dermal biopsy punch used to cut the radii. Great care was taken to ensure clean-cut samples, reducing sample flaws that could result in inaccurate testing. Placing the gage-length of the ‘dog-bone’ shaped samples between two glass-slides and measuring the thickness of the sample using a digital micrometer determined sample thickness. PCL, PES-PCL and PCL-PDMS samples were placed into aluminum grips and pulled at a cross-head speed of 50 mm/min by a 50-lb load cell to failure. Gelatin-PCL samples were soaked in phosphate buffered saline (PBS) solution for 1 h at 37°C , to simulate the rapid hydration and softening of the gelatin in aqueous solution, and pulled to failure immediately following the soak using the methods described above. The mechanical properties of core-shell PCL-Collagen, PCL-HA and PCL-Matrigel were measured likewise. Force as a function of displacement was recorded and converted to engineering-stress versus percent elongation for analysis.

2.2.4. Surface properties of PCL and core-shell nanofibers—Surface wetting properties were quantified using contact angle goniometry. PCL and core-shell nanofibers scaffolds plasma treated with air gas, as described previously, were cut into 5×1 cm segments and water contact angle was measured using a Krüss Easydrop (Krüss, Hamburg, Germany) water contact system. A 300- μ L drop of deionized water was placed on a dry area of the fiber, and using the Easydrop software, water contact angle was measured using a sessile drop contact to surface measurement. Five measurements were made and the average $\pm$ standard deviation (SD) was recorded.

2.2.5. Characterization of core-shell structure using transmission electron microscopy (TEM)—To examine the core-shell structure, polymer solutions were directly spun as described above onto TEM 200 mesh copper grids (Ernest F. Fullam, Inc., NY) for ~ 2 s to obtain representative PDMS-PCL core-shell nanofibers. These were then imaged using a transmission electron microscope (Tecnai 20, FEI Company, Hillsboro, OR).

2.3. Patient derived OSU-2 cell culture

Primary tumor cells were obtained from a patient with GBM under OSU approved IRB protocol 2005C0075 (dated 11/08/08). Written consent was obtained from all participants involved in this study. Patient-derived GBM (OSU-2) cells obtained from tumor tissue were cultured routinely as described previously [39]. Briefly, cells were cultured in DMEM/F12 (Invitrogen) containing 10% fetal bovine serum (Invitrogen) with 1% Pen-Strep (Invitrogen), fed 2–3 times a week and passaged on reaching confluency.

2.4. Analysis of cell adhesion on PCL and core-shell nanofibers

Scaffolds (electrospun nanofibers deposited on ~ 16 mm discs) were fixed using a medical adhesive (Dow Corning Silastic Brand, Medical Adhesive, Silicone Type A) to the bottom of a 24 well plate where the bottom was drilled (hole size ~ 11.2 mm dia). For sterilization, scaffolds were incubated with 70% ethanol combined with UV in a tissue culture hood (0.5 h), after which they were washed with PBS and allowed to dry overnight to remove any residual ethanol. Before cell seeding, scaffolds were washed with OSU-2 cell culture media (3 $\times$) and incubated for ~ 1 h. To examine initial cell adhesion, $\sim 20,000$ cells pre-labeled with cell tracker (CMFDA Invitrogen) were seeded on all scaffolds ($N = 3$, for each scaffold). After 0.5 h, scaffolds were washed with cell culture media (3 $\times$) and imaged using an inverted fluorescence microscope (Olympus IX 71) with a 10 $\times$ objective. Images were acquired at random locations ($n = 7$, for each well) and were thresholded to allow rapid quantification of cell adhesion using the particle analysis function in Image J. Cell adhesion to all scaffolds is reported as average number of cells adhered per unit area (mm^2) $\pm$ SD.

2.5. Morphological analysis of OSU-2 cells on PCL and core-shell nanofibers

Nanofiber scaffolds were sterilized as described previously (Section 2.4). Following this, OSU-2 cells, pre-labeled with a cell tracker dye (CMFDA Green, Invitrogen) were seeded on all scaffolds. After ~ 24 h, images of cells on nanofiber scaffolds ($N = 3$, for each scaffold) were acquired at random positions and subjected to image analysis. To examine the interaction of cells with nanofiber scaffolds, the Feret diameter (i.e., maximum distance between any two points in a region of interest, i.e., in this case, a single cell) was quantified

using Image J. This parameter provides the extent of elongation of a cell along the nanofiber. At least 140 individual cells were analyzed on each nanofiber scaffold examined and are reported as the average $\pm$ SD for all cells analyzed.

2.6. Analysis of single cell migration using time lapse confocal imaging on PCL and core-shell nanofibers

All scaffolds were sterilized as described in Section 2.4. OSU-2 cells, pre-labeled with a cell tracker dye (CMFDA, Green, Invitrogen) were seeded on scaffolds ($N = 3$) at $\sim 20,000$ cells/well in a 24 well plate. After ~ 6 h, scaffolds were washed with OSU-2 cell culture media ($3\times$) to remove non-adherent cells and then placed on the microscope stage equipped with a Weather Station (Precision Control LLC) to maintain incubator conditions for ~ 3 h prior to imaging. Following this, a $50\text{ }\mu\text{m}$ z-stack (step size = $10\text{ }\mu\text{m}$, no. of steps = 6) was captured at multiple random locations for each scaffold every 20 min for a total of 12 h using an inverted microscope (Olympus IX 71) equipped with a spinning disk confocal attachment. The z stacks were then projected (maximum) and concatenated to create migration movies. Movies were analyzed using the Image J MTrack J plug in. Cells that underwent proliferation during the time course of analysis were not considered for migration analysis (e.g., Movie S1). At least 95 individual cells for each substrate type were tracked using MTrack J. Migration speeds for all nanofiber scaffolds are reported as box and whisker plots showing median, mean, and outliers.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.03.069>.

2.7. Analysis of FAK/MLC2 signaling using western blotting on PCL and core-shell nanofibers

OSU-2 cells were cultured as described in Section 2.3. Approximately, 5×10^5 cells were seeded on pre-sterilized electrospun core-shell and PCL scaffolds glued to 60 mm culture dishes and cultured for ~ 48 h. After 48 h, OSU-2 cell-nanofiber constructs were frozen at $-80\text{ }^{\circ}\text{C}$. Following this, cells were lysed in 20 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 1% v/v NP-40, 0.5% v/v sodium deoxycholate, and protease plus phosphatase inhibitors (Complete and PhosSTOP cocktails; Roche Applied Science, Indianapolis, IN). Proteins were processed for Western blot analysis with antibodies against focal adhesion kinase (FAK), phospho-Tyr⁹²⁵ FAK (pFAK), myosin light chain 2 (MLC2), phospho-Ser¹⁹ MLC2 (pMLC2), and β -tubulin (all from Cell Signaling, Danvers, MA).

2.8. Statistical analysis

All data was analyzed using statistical analysis software (JMP Pro 9) by Oneway ANOVA. Statistical differences between nanofiber scaffolds post ANOVA were performed using the Tukey-Kramer HSD test. In all cases unless otherwise noted, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characterization of PCL nanofibers and core–shell nanofibers

PCL electrospun nanofibers exhibited submicron fiber diameters ($\sim 0.9 \mu\text{m}$) with cylindrical morphologies as observed via SEM (Fig. 1, Table 1). Nanofibers produced via core–shell electrospinning with an identical shell (PCL) but varying polymeric core materials exhibited microstructural characteristics very similar to PCL with diameters ranging from ~ 0.8 to $0.9 \mu\text{m}$ (Table 1) and fiber densities of ~ 570 – 650 fibers/mm (this line density translates to $\sim 19,000$ – $22,000$ fibers/ mm^2) (Table 1). These diameters and densities are within the range observed for white matter tracts *in vivo* (e.g., fiber diameter of ~ 0.5 – $3 \mu\text{m}$ and fiber densities of $\sim 10,000$ – $30,000$ fibers/ mm^2 [40,41]). Further, FFT analysis of these images showed symmetric, central narrow bands indicating nearly aligned nanofibers for all substrates investigated (Fig. 1, FFT images shown as insets). Radial summation of pixel intensities, followed by normalization further confirmed nearly uniform alignment for all samples examined, with two narrow peaks seen for all samples at 90° and 270° (because of symmetry) with a significant overlap of normalized intensity profiles. In a representative PDMS-PCL nanofiber, TEM revealed the presence of the expected core–shell structure (i.e., a PDMS ‘core’ surrounded by a PCL ‘shell’) (Fig. 2).

Despite these similarities in microstructure, PCL, and core–shell gelatin-PCL, PES-PCL and PDMS-PCL exhibited dramatically different moduli. The modulus (i.e., global modulus) of gelatin-PCL was the lowest (2.4 MPa) followed by PCL (7.9 MPa, $\sim 3\times$ that of gelatin-PCL), PES-PCL (28.6 MPa) and PDMS-PCL (33.3 MPa, both $\sim 4\times$ compared to PCL, the moduli of PES-PCL and PDMS-PCL not being dramatically different from each other). Ultimate tensile strength (UTS) for these nanofibers followed trends observed for the modulus (Table 1). These electrospun nanofibers with identical surface chemistry (i.e., PCL) exhibited nearly identical surface hydrophilicity as confirmed via contact angle goniometry (i.e., a contact angle of 0°) indicative of complete surface wetting following plasma treatment [42].

Similarly, nanofibers for chemical microenvironment investigations with identical core (i.e., PCL) and varying shell materials (i.e., HA, collagen, Matrigel) also exhibited similar microstructural features to pure, aligned PCL nanofibers: fiber diameters of ~ 0.8 – $0.85 \mu\text{m}$ and densities of ~ 600 – 700 fibers/mm (Table 2). Fiber alignment was confirmed via FFT analysis (Fig. 1). Further, complete water wetting was observed for these nanofibers. In addition, these materials exhibited similar moduli and UTS (Table 2), thus conserving mechanical properties while displaying different chemistries.

3.2. OSU-2 cell adhesion on PCL nanofibers and core–shell nanofibers

These scaffolds were used to investigate the influences of mechanics and chemistry on OSU-2 cell attachment. Initial adhesion was examined to avoid confounding influences from cell proliferation. In aligned core–shell nanofiber scaffolds presenting altered mechanical properties, no significant differences in initial cell adhesion were observed at the short time points investigated ($p = 0.1054$, ANOVA) (Fig. 3A, Table 3). In contrast, OSU-2 cells on nanofibers having identical PCL cores but varied shell surface chemistries showed

differences in cell adhesion ($p < 0.0001$, ANOVA). HA surface chemistry reduced cell adhesion compared to PCL. Surprisingly, this behavior was also observed for PCL-collagen. However, Matrigel did not significantly alter adhesion versus PCL (Fig. 3B, Table 3).

3.3. OSU-2 cell morphology on PCL nanofibers and core-shell nanofibers

Next, the morphological signatures of OSU-2 cells as a function of fiber mechanics and chemistry were explored. The Feret diameter of single OSU-2 cells was quantified as a measure of cell elongation. In almost all scaffolds examined, cells elongated along the nanofibers, exhibiting a bipolar, spindle shaped morphology, reminiscent of the morphology observed *in vivo* [30,31,43,44] (Fig. 4). However, the extent of elongation was influenced by the mechanical properties of the underlying nanofiber scaffold. Feret diameters were highest for fibers of intermediate modulus (i.e., $\sim 236 \mu\text{m}$ for $\sim 8 \text{ MPa}$ PCL) and lower for scaffolds of lower modulus (i.e., $\sim 169 \mu\text{m}$ for $\sim 2 \text{ MPa}$ gelatin-PCL) and higher modulus (i.e., $\sim 192 \mu\text{m}$ for PDMS-PCL and $\sim 200 \mu\text{m}$ for PES-PCL, both $\sim 30 \text{ MPa}$) with the lowest feret diameters seen on the softest nanofibers examined. This morphological behavior thus displayed sensitivity to intermediate nanofiber moduli (Fig. 5A, Table 3).

Similarly, extent of elongation was also influenced by nanofiber surface chemistry. In all chemistries examined, feret diameters were significantly lower than those of PCL ($p < 0.0001$) (Fig. 5B, Table 3). For PCL-collagen nanofibers, however, individual cell areas were comparable to those of PCL (not shown), indicating that cells on PCL-collagen were more likely to spread transversely possibly engaging multiple nanofibers. On PCL-Matrigel, OSU-2 cells were more likely to possess rounded morphologies extending some processes along the nanofibers.

3.4. OSU-2 single cell migration on PCL nanofibers and core-shell nanofibers

OSU-2 single cell migration was examined on PCL and other core-shell nanofibers using time-lapse confocal microscopy. Migration was sensitive to nanofibers with different mechanical properties. The fastest migration speeds observed for fibers of intermediate modulus (i.e., $\sim 11 \mu\text{m/h}$ for $\sim 8 \text{ MPa}$ PCL nanofibers, Movie S2), with slower migration speeds evidenced on both lower (i.e., $\sim 3.5 \mu\text{m/h}$ for $\sim 2 \text{ MPa}$ gelatin-PCL, Movie S3) and higher (i.e., $\sim 6.3 \mu\text{m/h}$ for PDMS-PCL and $\sim 5.8 \mu\text{m/h}$ for PES-PCL, both $\sim 30 \text{ MPa}$, Movie S4 and, Movie S5, respectively) moduli nanofibers (Fig. 6A, Table 3). Thus, this ENF system demonstrated for the first time that glioma migration, in addition to being a function of modulus, can exhibit a peak value at a particular substrate modulus. Further increases in the modulus (i.e., $\sim 30 \text{ MPa}$ PES-PCL or PDMS-PCL versus $\sim 8 \text{ MPa}$ PCL) reduced migration speed, although these speeds were still significantly higher ($p < 0.005$, Tukey-Kramer HSD) than those observed with the softest core-shell nanofiber (i.e., $\sim 2 \text{ MPa}$ gelatin-PCL). These trends are also in agreement with the observed morphological responses (i.e., Feret diameter). Interestingly, in no case was cell migration completely inhibited, further demonstrating that aligned topographical features of the appropriate scale are sufficient to promote cell migration. However, tumor cell migration speed was a strong function of nanofiber mechanics.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.03.069>.

In addition to mechanics, core-shell nanofibers with various surface chemistries (i.e., HA, Collagen-I, and Matrigel) with a PCL core were examined. In this case, cell migration was sensitive only to PCL-HA core-shell nanofibers (Movie S6), demonstrating significantly decreased cell migration speeds compared to PCL nanofibers ($p < 0.0001$, Tukey-Kramer HSD) (Fig. 6B, Table 3). Further, cell detachment can be visualized during the time course of the movie, consistent with reduced cell attachment observed in Fig. 3. Thus, HA acts as a negative chemical cue to both adhesion and migration in this setting. Interestingly, collagen-I (Movie S7) and Matrigel (Movie S8) surface chemistries on PCL core nanofibers had no significant effect on cell migration compared to bare PCL nanofibers ($p > 0.2$, Tukey-Kramer HSD). Thus, in contrast to observations with nanofibers with altered mechanics, migration results did not correlate strongly with morphological observations except the case of PCL-HA. This may occur because, although cells less elongated on PCL-collagen/PCL-Matrigel versus PCL, they may have interacted with multiple fibers, enhancing migration potential.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.03.069>.

3.5. FAK/MLC2 signaling expression on PCL nanofibers and core-shell nanofibers

To further evaluate the mechanisms behind the observed migration responses in fibers with different mechanical moduli, we investigated the expression of molecular markers for cell adhesion (Focal Adhesion Kinase, FAK) and migration (Myosin Light Chain 2, MLC2). Both FAK [45] and MLC2 (Isoform A) [46] are overexpressed in GBM tissue. Consistent with single cell migration results, the active (i.e., phosphorylated) forms of FAK and MLC2 were overexpressed by cells on fibers that supported more rapid migration compared to those where cell adhesion, elongation, and migration were hindered, such as gelatin-PCL (Fig. 7A, Table 3). In fact, reduced cell migration on gelatin-PCL correlated not only with reduced activation of FAK and MLC2, but with reduced total expression of these proteins, suggesting considerably reduced formation of adhesion complexes and engagement of cytoskeletal motors on this substrate. Expression of these markers in cells cultured on nanofibers with moduli greater than PCL (i.e., PES-PCL and PDMS-PCL) was slightly lower, but comparable to PCL.

Since the HA 'shell' chemistry produced a significant reduction in migration potential compared to PCL, expression FAK and MLC2 was also examined using this substrate. Interestingly, total FAK and MLC2 expression was not significantly different on PCL and PCL-HA, in stark contrast with the result observed for gelatin-PCL. However, pFAK and pMLC2 were significantly reduced on PCL-HA (Fig. 7B, Table 3), indicating that reduced cell adhesion/migration correlated well with reduced activation of these markers. In sum, overall expression of FAK and MLC2 positively correlated with the regulation of GBM cell migration triggered by nanofiber mechanics, whereas specific chemistries (i.e., HA) correlated with regulation of the phosphorylated forms only and not total expression. Thus, in this model, both mechanics and chemistry play crucial roles in regulation of cell

migration behaviors, possibly through different regulatory schemes. Further, chemistry was shown to exert an independent influence on migration from mechanics (modulus of PCL-HA nanofibers = 7.6 ± 1.2 MPa, not different from modulus of PCL nanofibers = 7.9 ± 1 MPa).

4. Discussion

In this study, aligned electrospun nanofibers that mimic the topographical features of white matter were fabricated via core-shell electrospinning. Using this approach, nanofibers with different mechanical properties, yet nearly identical micro-architectures and surface chemistries, were prepared. Similarly, core-shell nanofibers displaying different surface chemistries on an identical core (i.e., PCL) nanofiber were also fabricated to maintain nearly consistent mechanical properties while altering surface chemistry. The core-shell spinning methodology thus provides an elegant means of isolating the effects of individual factors (e.g., mechanics, chemistry) on cell behaviors in a microenvironment that mimics some features of *in vivo* topography as observed here and as demonstrated previously [30,31]. In contrast, decoupling the physical (e.g., porosity, mechanics) and chemical properties of 3D hydrogels is relatively challenging, limiting the ability to independently investigate the effects of these variables on tumor cell behaviors. For example, in most mechanical studies using 3D hydrogel systems, modulus is adjusted by altering the polymer density, which introduces changes in chemistry as well as alteration in adhesion site density. Similarly, in other electrospun polymeric systems, modulus is altered by varying polymer composition, which may be associated with changes in microstructure and chemistry. This work thus addresses a crucial issue in brain cancer biology by providing tunable and well defined platforms that mimic a major GBM migration highway (i.e., white matter tracts) while simultaneously allowing examination of biophysical and biochemical cues regulating tumor cell behaviors. Further, in comparison to core-shell nanofibers spun previously in random orientations (i.e., gelatin-PCL [47], PES-PCL [38], PCL-collagen [48]), this work employed aligned core-shell nanofibers and introduced new biomaterials (i.e., PDMS-PCL, PCL-HA and PCL-Matrigel core-shell nanofibers), which may have applications in neural tissue engineering.

Using core-shell electrospinning, nanofiber modulus was altered to examine the effect of mechanics on GBM cell behaviors. Nanofibers fabricated displayed moduli ranging from ~2 to 30 MPa. Few reports have examined the mechanical properties of myelinated central nervous system axons/nerve fibers, primarily using atomic force microscopy (AFM) [49]. Properties measured using this approach display a similar order of magnitude range to our electrospun nanofibers. Although cells may sense local modulus, and experimental values for local modulus may provide further insight, the bell-shaped response observed here, with optimal migration at intermediate modulus, shows that this global modulus range captures biologically-relevant migration behavior better than TCPS/glass. Thus, for the first time, a peak in migration speed was observed (e.g., PCL, ~ 8 MPa). Cells on nanofibers exhibiting moduli less than that of PCL (i.e., ~ 2 MPa) and greater than PCL (~30 MPa) displayed slower migration, with the softest nanofiber examined promoting the slowest migration. This may result from a lowered resistance to cell-generated traction forces, as has been reported on previously on soft substrates. Further, sensitivity to intermediate nanofiber modulus

correlates with a known cellular sensing process referred to as the “catch-bond formation” mechanism [50] in which cell interaction with the underlying substrate is significantly enhanced at a particular rigidity level – in our case, ~ 8 MPa. This mechanical sensitivity in migration was not directly correlated to initial, short-term cell adhesion. Thus, initial cell adhesion is most likely guided by the surface properties of the underlying nanofiber scaffold and not mechanical features or chemical features, which were fixed.

Migration results were further validated by studies of FAK/MLC2 signaling. The lowest expression levels of these proteins were seen on the softest fibers, gelatin-PCL, correlating to adhesion and migration data. Whereas involvement of these pathways in glioma migration has been implicated previously [45,46,51], this is the first study to show a strong dependence of these pathways on nanofiber modulus. These results suggest that GBM cell behaviors including feret diameter, migration speed, FAK and MLC2 expression, are all tightly regulated by the mechanical properties of the underlying nanofiber scaffold.

In contrast, results from fibers with altered surface chemistries, but nearly identical moduli demonstrated only marginal influence on migration speed. Whereas each chemistry elicited unique responses either in terms of morphology or adhesion, HA was the only chemistry that significantly influenced (i.e., impeded) cell motion in an aligned topography-mimetic setting. This correlated well with observations of reduced initial cell adhesion, reduced feret diameter, and reduced expression levels of pFAK and pMLC2 observed for PCL-HA versus the PCL control. These observations are in agreement with the chemo-repellant role of HA *in vivo* [52], and our previous *in vitro* observations of GBM (OSU-2) migration behaviors in HA-based hydrogel systems [14]. Importantly, our findings using HA in a topography-mimetic setting are in stark contrast with a migration-inducing role of HA, observed when this glycosaminoglycan is used as a soluble factor or incorporated with other ECM components [53–55]. In agreement with observations using HA-hydrogel systems [15], our findings strongly suggest that the physical presentation of HA plays a critical role in migration. Thus, this biomimetic materials platform, mimicking *in vivo* topography and HA chemistry, should enable further investigation of intracellular signaling pathways and associated events, in addition to those of the FAK/MLC2 pathways investigated here.

In contrast, other surface chemistries explored (i.e., PCL-Matrigel and PCL-collagen nanofibers) altered the morphology of OSU-2 cells, but not migration behaviors. Initial cell adhesion, cell area, and feret diameter (i.e., alignment to the scaffold) were equivalent to or less than that of the PCL control for cells cultured on both PCL-Matrigel and PCL-collagen nanofibers. Similar results for aligned PCL versus PCL blended with collagen nanofibers have been reported using the U-373 astrocytoma cell line [23]. In no case were cell attachment or spreading enhanced by altered surface chemistry, despite the fact that these chemistries are known to provide enhanced adhesive cues compared to the bare PCL nanofiber control (e.g., [48]) and we have observed a migration promoting role of collagen in hydrogel form [14]. It is important to note that short-term adhesion (0.5 h) was examined to minimize confounding influence from proliferation versus longer time points (i.e., 24 h), which might have yielded different results. This suggests that alternative pathways/mechanisms could be at play in supporting cell movement versus adhesion, and that

topographical cues alone could be sufficient to support migration. Detailed interrogation into the adhesion and migration mechanisms should provide further insight into such behaviors.

Collectively, these results demonstrate that core-shell electrospun nanofibers permit examination of cell behaviors in response to specific, individual, chemical, mechanical, or topographical cues that may mimic features of 3D migration. In general, ENF porosity is insufficient to permit cell migration without modification [56], and thus cell culture is limited to their surfaces (e.g., 2D). However, individual tumor cells migrate differently on electrospun nanofibers versus a flat surface (e.g., 2DTCPS), with the scaffold being sufficiently irregular for cells to exhibit 3D migratory patterns (e.g., elongation along fiber length and formation of protrusions along the fiber that closely mimic cell protrusions through a 3D porous hydrogel matrix). The ability of aligned electrospun nanofibers to mimic the morphological, and in part, molecular signatures, of 3D migration (e.g., migration driven by myosin-II) comparable to those seen in brain slices has been demonstrated previously [30,31]. This work examines the role of mechanics and chemistry on tumor cell behaviors and associated signaling pathways, thereby establishing a new model to analyze GBM migration at the single cell level and its validation with molecular markers. Further, these biomaterials could be broadly applied to study behaviors of neural cells in white matter mimetic microenvironments with potential implications for stem cell therapy, studies of neural regeneration and development, and myelination.

5. Conclusions

Here, we developed and characterized a white matter tract, topography-mimetic, aligned ENF platform fabricated via core-shell electrospinning. This platform displayed independently tunable mechanical and chemical properties, thus providing the potential to further explore the complex interplay of mechanics, chemistry and topography on malignant tumor cell behaviors. We have demonstrated its utility in studying patient-derived GBM behaviors, including cell adhesion, morphology, migration and FAK/MLC2 signaling expression, revealing GBM sensitivity to nanofiber mechanics, as well as surface chemistry. This biomimetic material platform should have broad applicability for examining and further evaluating fundamental questions in the biology of brain tumors as well as in neuroscience *ex vivo*. Finally, these mechanical and chemically tunable biomaterials could also be employed to examine behaviors of other types of tumor cells (e.g., prostrate, head and neck tumors) that metastasize via perineural structures [57] and cell behaviors of other tissues having similar topographical architectures (e.g., the aligned myocardium in the heart [58]) thereby demonstrating its broad utility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Micro-structural features of PCL and core-shell nanofibers observed via scanning electron microscopy (SEM). (A) Gelatin-PCL (B) PCL (C) PDMS-PCL (D) PES-PCL (E) PCL-Collagen (F) PCL-HA (G) PCL-Matrigel. Scale bar indicates 20 µm. Fast Fourier Transform (FFT) analyses of the associated images are shown as insets. (H) FFT analysis via radial summation of pixels normalized for all samples and plotted versus degree.

Fig. 2. Representative PDMS-PCL core-shell nanofiber. (A) Schematic and (B) transmission electron microscopy (TEM) image of the PDMS-PCL core-shell nanofiber showing the PDMS 'core' and PCL 'shell' surrounding the core indicated by white bidirectional arrows. Scale bar = 0.2 μm .

Fig. 3.

Initial attachment of OSU-2 cells to nanofiber scaffolds. (A) Adhesion as a function of nanofiber mechanics and (B) Adhesion as a function of surface chemistry. * indicates statistically significant difference compared to PCL nanofiber control ($p < 0.05$).

Fig. 4. OSU-2 cell morphology on nanofiber scaffolds. (A) Gelatin-PCL (B) PCL (C) PDMS-PCL (D) PES-PCL (E) PCL-Collagen (F) PCL-HA (G) PCL-Matrigel. Scale bar in (A) indicates 100 μm . Bidirectional arrow indicates direction of fiber alignment.

Fig. 5. Feret diameter analysis of OSU-2 cells on various nanofibers. (A) Feret diameter as a function of mechanics and (B) Feret diameter as a function of biomimetic chemistries. *N* 142 individual cells analyzed for each nanofiber. * and ** indicates statistically significant difference compared to PCL nanofiber ($p < 0.05$). Levels marked by identical number of * are not significantly different from each other as determined by Tukey-HSD test.

Fig. 6. Single cell migration speeds on electrospun nanofiber scaffolds. (A) Migration as a function of nanofiber mechanics and (B) migration as a function of surface chemistry shown in box and whisker plots. $N = 95$ individual cells analyzed for each nanofiber. * and ** indicate statistically significant difference compared to PCL nanofibers ($p < 0.05$). Levels marked by identical numbers of * are not significantly different from each other as determined by the Tukey-HSD test.

Fig. 7. Analysis of FAK/MLC2 expression via western blotting. (A) pFAK, FAK, pMLC2, MLC2 expression as a function of mechanics. (B) pFAK, FAK, pMLC2, MLC2 expression for PCL versus PCL-HA electrospun nanofibers. In both cases, tubulin served as a loading control.

Table 1

Microstructural and mechanical characterization of PCL and core-shell nanofibers with altered cores and an identical surface chemistry (PCL). All nanofibers examined displayed a contact angle of 0°, indicative of complete wetting.

Scaffold	Fiber diameter (nm)	Fiber density (mm ⁻¹)	Modulus (MPa)	UTS ^a (MPa)
Gelatin-PCL	880 ± 295	577 ± 151	2.4 ± 0.6	0.8 ± 0.2
PCL	925 ± 183	594 ± 100	7.9 ± 1	4.6 ± 1.3
PES-PCL	854 ± 302	648 ± 159	28.6 ± 6.6	9.2 ± 1.7
PDMS-PCL	829 ± 232	638 ± 94	33.3 ± 6.9	13.1 ± 2.2

^a Ultimate Tensile Strength.

Table 2

Microstructural and mechanical characterization of core–shell nanofibers with altered surface chemistries and an identical core (PCL). All nanofibers examined displayed a contact angle of 0°, indicative of complete wetting. Data for PCL (from Table 1) is included for comparison to other core–shell nanofibers.

Scaffold	Fiber diameter (nm)	Fiber density (mm ⁻¹)	Modulus (MPa)	UTS ^a (MPa)
PCL	925 ± 183	594 ± 100	7.9 ± 1	4.6 ± 1.3
PCL-Collagen	841 ± 329	622 ± 145	6.4 ± 1.3	3.6 ± 0.8
PCL-HA	813 ± 246	597 ± 106	7.6 ± 1.2	3.8 ± 0.4
PCL-Matrigel	815 ± 300	684 ± 101	7.8 ± 1	2.5 ± 0.6

^a Ultimate Tensile Strength.

Table 3

Changes in initial cell attachment, cell stretching, total migration and protein expression on core-shell nanofibers as a function of mechanics and chemistry as compared to PCL nanofiber controls.

Parameters compared to PCL control		Protein expression					
Scaffold core	Scaffold shell	Initial attachment	Cell stretching	Total migration	pFAK	FAK	MLC2
Mech	Gelatin	NC ^a	↓	↓	↓	↓	↓
	PES	NC	↓	↓	NC	Slight ↓	NC
	PDMS	NC	↓	↓	NC	NC	NC
Chem	PCL	↓	↓	↓	↓	NC	NC
	Collagen	↓	↓	NC	-	-	-
	Matrigel	NC	↓	NC	-	-	-

^aNo Change.