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PAPER

Tandem electrospinning for heterogeneous nanofiber patterns

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Abstract

Smart nanofibrillar constructs can be a promising technological solution for many emerging and established fields, facilitating the potential impact of nano-scale strategies to address relevant technological challenges. As a fabrication technique, electrospinning (ESP) is relatively well-known, accessible, economic, and fast, though until now has shown limitation over control and design of the fibrillar constructs which can be produced. Here, we introduce 'Tandem Electrospinning' (T-ESP), a novel technique able to create increasingly complex patterns of fibrous polymeric constructs on a micro and nano-scale. Modifying a standard ESP configuration results in a focusing electric field that is able to spatially define the deposition pattern of multiple polymer jets simultaneously. Additional spatially defined heterogeneity is achieved by tuning polymer solution properties to obtain a gradient of fiber alignment. Heterogeneous fibrous meshes are created with either random, aligned, or a divergent fiber patterns. This approach holds potential for many fields, with application examples shown for tissue engineering and separation technologies. The technique outlined here provides a rapid, versatile, and accessible method for polymeric nanofabrication of patterned heterogeneous fibrous constructs. Polymer properties are also shown to dictate fiber alignment, providing further insight into the mechanisms involved in the electrospinning fabrication process.

Introduction

Recent years have seen a renewed interest in electrospinning (ESP) for micro- and nano-scale fabrication, driven by new applications in the fields of environment and security, such as filtration or particle sensing, biomedical technologies, in form of tissue scaffolds or drug release platforms, and in the energy sector, with potential for improving both solar and fuel cell technologies [1–6]. The promise of creating micro- and nano-sized fibers in a simple, relatively inexpensive and high-throughput manner has spurred

development of ESP fabrication techniques, leading to the production of fibrous meshes with high surface area and tunable porosity.

Conventional ESP results in a chaotic fibrous mesh, narrowing the application of this form of ESP to areas such as textile and filtration industries. Strategies have been developed to produce fiber constructs useful for other applications, but these approaches still exhibit limitations in terms of spatially defined fiber deposition and orientation, and typically produce simple polymer constructs consisting of a single fiber type. ESP across a planar target gap electrode or

patterned target electrodes produces simple parallel fibers or cross-hatched fiber patterns, respectively, with limited spatial definition and restricted to single fiber composition or layer-by-layer fiber assemblies [1, 7–11]. In general, deposition and spatial control of multiple polymer fibers is hampered by the mutual repulsion of multiple charged jets [12–14]. Attempts to create multimodal fiber constructs have relied on the chaotic fluctuations of the ‘whipping’ polymer jets to achieve overlap between fiber populations, intrinsically precluding any form of fiber orientation. ESP onto a rotating mandrel also produces simple parallel fibers, with increased complexity possible by simultaneously electrospinning different polymers onto a rotating mandrel [15]. However, more elaborate, spatially defined multimodal patterns are not possible. The most detailed fiber patterns have been created by near-field electrospinning (NFES) or melt electrospinning, which are less accessible and unable to simultaneously deposit multiple fiber types within a confined region [16, 17].

Here, we describe the development of T-ESP techniques, which allow for simultaneous electrospinning of up to three polymer fibers. Using modifications to a traditional ESP setup, we can achieve a high degree of control of planar ESP patterning. Control over multiple jets is achieved by manipulating the electrostatic field, resulting in the creation of ESP multi-material fibrous constructs. This allows ESP fiber patterns of intermingled, heterogeneous fiber populations in random, parallel or divergent (‘Y-shaped’) orientations. Finally, we show how this facile and economic fabrication method can be used to mimic branched structures found in biological systems and present an example of a divergent tissue scaffold supporting spatially defined differences in neurite outgrowth.

Experimental section

Polymer solution preparation and characterization

All polymers were dissolved in associated solvent solutions overnight. The PEOT/PBT polymers were prepared in a solvent blend of chloroform and hexafluoroisopropanol (HFIP; see supplementary material, tables S1 and S2 are available online at stacks.iop.org/BF/12/025010/mmedia for a details on polymers and solutions) and appropriate dyes were added at 0.01% w/v. Specifically, a 20% w/v solution of 300PEOT55PBT45 polymer was prepared and designated HighE for its small molecular entanglement weight (M_e) of 250 Da, a 20% w/v solution of 300PEOT70PBT30 was prepared and labelled MidE (M_e : 450 Da), and polymer solutions of 1000PEOT70PBT30 (M_e : 710 Da) were prepared at either 20% w/v (LowE) or 15% w/v (LowE_{mv}; mv: matched viscosity). Where applicable, NaCl (Sigma Aldrich GmbH, Germany) was added to polymer solutions at final concentration of 0.05 mg ml⁻¹ and

polymer solutions were labelled with a ‘+’ (e.g. LowE⁺). Conductivity was measured using a custom gold parallel plate apparatus at 20 °C (see supplementary material S12 for full description) and viscosity was measured using a Brookfield DV-E with an s21 mandrel at 30 °C and 100 rpm.

Electrospinning for T-ESP development

A custom ESP setup was used with an environmental chamber (25 °C, 30% humidity) and a large upper parallel plate (30 cm by 20 cm). ESP was performed at 25 KV, a working distance of 20 cm, a flow rate of 1 ml h⁻¹ and for an ESP interval of 1 min. For tandem ESP and triple ESP, needles were mounted along the Y axis of the collector target (figure 1(B)) at an inter-needle separation of 1 cm and 5 cm, respectively. A 14 mm coverslip (Menzel-Glaser) was the target substrate used to collect deposited fibers, placed on the collector electrodes of either a parallel or epsilon arrangement (see supplementary figure S8).

Electrospinning T-ESP scaffolds for specific applications

Cell scaffolds were prepared using the epsilon electrode configuration shown in Figure S9. In lieu of coverslips for improved cell culture handling, fibers were deposited onto a mesh support ring with an outer diameter 15 mm and an inner aperture diameter of 12 mm, made from a nonwoven sheet of polyester (6691 LL (40 g m⁻²), a kind gift from Lantor B.V., the Netherlands). A working distance of 20 cm and a voltage of 20 KV was used for an ESP time of 30 s. To ensure jet stability, the HighE solution had a flow rate of 1 ml h⁻¹ and a flow rate of 0.5 ml h⁻¹ for the HighE-collagen solution. Parallel T-ESP scaffolds for liquid phase separation were prepared with the standard gap electrode configuration using an HighE solution (1.0 ml h⁻¹) and a 10% PVA solution (0.5 ml h⁻¹) on glass coverslips. Scaffold were immersed in methanol with 5% paraformaldehyde for 24 h to crosslink the PVA fibers and then air dried for 24 h before use.

Analysis of ESP process

Images of whipping were captured by a Luminix DMC G3 and fiber pattern images were stitched together using a Nikon Eclipse Ti with an automated stage at 10× magnification. Fibers were gold sputter-coated and examined with a XL 30 ESEM-FEG (Phillips) operating at 10 kV. A minimum of 100 fibers were measured per population and minimum of 5 images per fiber population were evaluated for fiber orientation using the OrientationJ plugin [18], providing a coherence value between 0 (isotropic) and 1 (perfectly anisotropic). Fiber orientation was also evaluated by creating an FFT image of SEM images [19].

Cell culture

Dorsal root ganglia (DRGs) were explanted from 2 d old rat pups (Wistar Unilever: HsdCpb:WA). All procedures followed national and European laws and guidelines and were approved by the local ethical committee. Briefly, rats were sacrificed by cervical dislocation under general anaesthesia (4% Isoflurane) and then decapitated. Individual ganglia were removed from the spinal column and nerve roots were stripped under aseptic conditions with the aid of a stereomicroscope. DRGs were cut in half to expose interior neurites and placed on the fibrous ESP scaffolds. For divergent T-ESP scaffolds, the DRG was placed at the divergent junction of the fibrous bifurcation. DRGs were cultured in NeuralBasal A- medium with B27 supplement, 0.5 mM L-glutamine and pen/strep added (Gibco/Invitrogen) and 100 ng ml⁻¹ NGF (Sigma Aldrich). Cultures were maintained for 5 d, with medium refreshed after Day 1 and Day 3. Cells were fixed with ice cold 2% paraformaldehyde (PFA) for 15 min at 4 °C, then permeabilized for 10 min with

0.1% TritonX-100. Cultures were blocked for 1 h in 5% Normal Goat Serum and 1% BSA in a TRIS buffered solution (TBS), followed by a 16 h incubation with a mouse anti-b3-tubulin primary antibody (1:1000, Abcam), a triple wash in 1% BSA TBS solution and a 16 h incubation with an Alexa 594 anti-mouse secondary antibody (1:1000, Invitrogen) with 1% Normal Goat serum. Scaffolds were then washed and mounted with Mowiol 4-88 with 2.5% DABCO.

Neurite measurement

Fluorescence imaging of mounted DRG samples was performed with a BD Pathway 435 using a 10× objective. Automated Sholl Analysis was performed using ImageJ and the Sholl Analysis Plugin (ver.4.0.0) with a 20 μm step size between concentric shell measurements. The resulting output was used to determine the 10 longest neurites, from which the average maximum length per sample was calculated.

Additional methodology information is available in supplementary material section.

Results and discussion

Electrode configuration focuses multiple polymer jets for controlled overlapping fiber deposition

Traditional ESP process can be described as an electrostatic extrusion of a charged polymer solution from a supply needle towards a grounded collector. During this process, a polymer jet is formed which is initially straight but then enters an unstable whipping phase. This chaotic motion further extrudes the polymer jet and accelerates solvent evaporation to form solid polymer fibers [20]. To achieve more control over fiber deposition, this study manipulated the electrostatic field by using a grounded target comprised of millimeter wide electrodes with a centimeter-wide gap (figure 1(A), Inset: 'Collector'). The electric field across the gap is known to exert forces on the fibers during the whipping phase (figure 1(D), Inset: 'Gap'), leading to fiber alignment across the gap [21]. However, the use of millimeter wide electrodes in this configuration also achieved a focusing effect of the electric field in the orthogonal plane (figure 1(C)) to further direct the evolution of the whipping phase.

ESP of one polymer solution with this arrangement led to the spatially confined deposition of aligned fibers across this gap (supplementary figure S2). Yet, this novel electrode architecture produced an electric field able to confine the simultaneous deposition of two ESP jets (figure 1(A), Inset 'Tandem Jets') to produce aligned parallel deposition of different fiber populations (figure 1(E)). To the best of our knowledge, this is the first report of concurrent and controlled deposition of different populations of aligned fibers with a well-defined region of overlap (figure 1(F)). When using optimized polymer solutions, as discussed below, this deposition was robust and repeatable with defined multi-fiber constructs consistently produced.

Despite initial mutual repulsion of the two jets during the stable jet phase (figure 1(A) Inset: 'Tandem Jets'), the focused electric field created by the narrow electrode geometry (figure 1(C)) provided sufficient force upon the fibers during the whipping phase to cause colocalized deposition. Fluorescence microscopy (figure 1(F)) revealed that the two regions were overlapping with no distinct boundary region between fiber populations. The fibers showed a relatively homogeneous diameter and were well aligned, with no statistically significant differences between populations.

Polymer properties dictate alignment for gap electrode ESP

The degree of fiber alignment achieved was found to be dependent on the properties of the polymer solution. This provided the possibility of further control over fiber deposition and degree of multi-modal complexity achievable.

The polymer solution has a dramatic impact on the ESP process in general, with many variables having a degree of interdependence. For example, the chosen solvent affects the solution viscosity but also the rate of evaporation during fiber formation, both of which alter ESP kinetics. Meanwhile, the polymer composition and molecular weight dictate the degree of solubility in a chosen solvent, the viscosity of the polymer solution, and can influence the rate of solvent evaporation [22].

To evaluate the impact of the solution properties on the T-ESP process, three copolymers from the PEOT/PBT family were selected. These polymers were synthesized using poly(ethylene oxide) (PEO) and poly(butylene terephthalate) (PBT) segments of specific lengths added in known proportions to produce a randomly distributed block copolymer. The PEO segment becomes terephthalated during the copolymer reaction and imparts a hydrophilic, amorphous phase, while the PBT introduces a hydrophobic, crystalline phase, such that the polymer properties can be tuned by controlling the length of the initial PEO segments and varying proportions of PEOT to PBT [23–25]. For example, a polymer with initial PEO segments of 300 Da and a PEOT/PBT of 55/45 has the designation of 300PEOT55PBT45. Most importantly, this class of polymer is known to be soluble in the same solvent solution (CHCl_3 and HFIP) so that the final solutions are similar with respect to evaporation rate, polymer/solvent interactions, and solvent conductivity. Furthermore, different polymer compositions are available at similar molecular weights of approximately 50 kDa, providing a relatively consistent basis for comparison.

A distinguishing characteristic of the different selected polymers was the entanglement molecular weight (M_e); this is a measure of the chain length between physical entanglement points of polymer chains in a solid bulk polymer. The three polymers selected were: a high entanglement (HighE) polymer with a composition of 300PEOT55PBT45 and a small M_e of 250 Da; a midrange molecular entanglement (MidE) polymer (300PEOT70PBT30; M_e : 450 Da); and 1000PEOT70PBT30, with an M_e of 710 Da and therefore called here LowE (low degree of molecular entanglement) [26]. These were prepared as 20% w/v polymer solutions in a solvent blend of 70% chloroform and 30% hexafluoroisopropanol (HFIP; see supplementary material, tables S1 and S2 for details on polymers and solutions used).

Despite similar solvent and polymer concentrations, the HighE, MidE and LowE polymers solutions demonstrated different viscosities of 120, 125 and 388 centipoise (cP), which produced fiber diameters of 0.86 ± 0.28 , 0.74 ± 0.34 , and 1.03 ± 0.35 μm respectively. Polymer solution viscosity is an indication of the degree of molecular entanglements in solution (not in bulk) [27]. M_e , along with viscosity and solution conductivity, has been cited as an important

parameter in describing the polymer solutions used for ESP [28–30]. To eliminate this difference in polymer solution molecular entanglement as a potential variable, we included a LowE polymer solution with the same molecular weight but a lower polymer concentration to achieve a similar viscosity of 122 cP (referred to as LowE_{mv}, ‘matched viscosity’; see table S2).

Unexpectedly, image analysis of fiber orientation found statistically significant decrease ($p < 0.01$) in alignment with increasing values of M_e . Using a score from 0 to 1 (random to aligned), alignments of 0.773 ± 0.089 , 0.641 ± 0.102 , and 0.33 ± 0.077 were measured for HighE, MidE, and LowE fibers, respectively. Consistent with this trend, the LowE_{mv} also resulted in low fiber alignment (0.222 ± 0.0698), indicating that viscosity, thus entanglement in the polymer solution, was not an effective predictor of fiber alignment. Instead, M_e as a bulk polymer property appeared to be the predominant factor. Until now, M_e has been used to describe the viscoelasticity of the polymer solution as it relates to the formation of ‘beads-on-a string’ fiber morphology formed in the initial stages of the polymer jet [28]. This finding suggested a potential role of M_e on fiber evolution after they have returned to a bulk polymer state later in the whipping phase, with increasing bulk entanglement (HighE) improving electric field-induced fiber alignment.

M_e affects whipping phase evolution and fiber alignment

Correlated with differences in fiber alignment, there were also observed changes in the whipping phase of the different polymers. The size and shape of the fiber deposition pattern onto the collector is direct evidence of how the whipping phase develops during the electrospinning process. The highly entangled HighE polymer deposited fibers along the length of the electrodes, indicating a smooth, elongated progression of the whipping phase. In comparison, the LowE polymer had fewer fibers along the length of the electrodes and a wider, more circular deposition in the electrode gap region, suggesting an abrupt, more focused evolution of the whipping fiber (supplementary material figure S1).

To account for this observed correlation between fiber alignment, macroscopic deposition pattern, and bulk molecular entanglement, it is proposed here that the fiber mechanical properties (as dictated by M_e) influence both fiber formation and the overall evolution of the whipping phase. While the whipping instability begins with an initial perturbation (bending) of the polymer jet, it has been reported that the fiber can also experience secondary and tertiary bending during the whipping phase. The consequence is that fibers are not necessarily smooth and straight, but can have a wavy, corrugated appearance [20]. It is

proposed here that a fiber with a lower degree of bulk entanglement (a higher M_e) experiences more plastic deformation and accumulates non-axisymmetric residual stress during this final whipping phase [31–33], leading to a curled, corrugated fiber. In comparison, a fiber of a highly entangled polymer would have increased elasticity, resisting deformation, and would incur less residual stress to produce a straight fiber morphology. It should also be remarked that the long working distance used (20 cm) excludes the curled fibers to be the result of buckling [34].

We suggest that the electric field imposes orientation along the length of the fibers, such that a general ‘global’ alignment is achieved; this is evidenced by the fact that the observed fiber orientation is not entirely random, especially compared to fibers deposited on a flat collector plate (see table S5). However, the resulting microscopic alignment of the fiber also depends on how corrugated the fibers have become, with higher M_e polymers producing fibers with some degree of alignment but with increasing disorder. Since strain and entanglement can also be affected by differences in molecular weight, the use of these particular polymers proved essential in identifying the role of M_e [35].

These additional plastic perturbations during fiber formation are also suggested to disrupt the whipping profile development, accounting for the observed differences in the elongated deposition of fibers. An elongated whipping profile may also increase the degree of microscopic fiber alignment of the HighE polymer fibers by improving global fiber alignment and effectively stretching out any fiber perturbations.

Heterogeneous fiber patterns can be tuned by adjusting polymer solution properties

T-ESP was extended to produce heterogeneous fiber populations, using the HighE and the LowE polymers as extremes in terms of both fiber size and fiber alignment. Again using a score from 0 to 1 (random to aligned), heterogeneous T-ESP produced the expected regions of aligned HighE fibers (0.699 ± 0.104), less aligned LowE fibers (0.401 ± 0.06), and an overlapping region between the two populations. Unexpectedly, this also resulted in a fiber deposition pattern with an extreme spatial bias, where the LowE fibers now shifted to the edge of the deposition region (figure 2(A)).

Aiming to understand this bias, a comparison was made between the profiles of the tandem jets when two identical HighE solutions were used or when HighE and LowE polymer solutions were tandem-spun together. This revealed that T-ESP with identical polymer solutions produced stable jets of equal length (figure 1(A) Inset: ‘Tandem Jets’) while heterogeneous T-ESP exhibited different lengths, with the HighE solution initiating the chaotic whipping phase at an earlier stage (i.e. a shorter stable jet) compared to the LowE solution (figure 2(B)). Because the whipping

Figure 2. Heterogeneous Tandem ESP Scaffolds. (A) Tandem ESP fibers of HighE (green) and LowE (red) produced a biased pattern of fiber deposition, though an overlapping gradient region between the two fiber populations was still maintained (inset). (B) The whipping jet profiles reveal that the LowE solution initiates whipping later compare to the HighE solution (scale bar: 5 cm). (C) The HighE fibers had a diameter of $0.723 \pm 0.261 \mu\text{m}$ and were well aligned (0.699 ± 0.061). (D) LowE fibers were significantly larger in diameter ($1.61 \pm 0.345 \mu\text{m}$, $p < 0.01$), and were less aligned (0.397 ± 0.076 , $p < 0.01$). (E) Tandem electrospun fibers of HighE polymer solution and LowE⁺ solution produced a centered pattern of fiber deposition with an overlapping gradient region between the two fibers also evident (inset). (F) The whipping profiles show that the HighE jet (left) and the LowE⁺ jet have similar stable jet lengths (scale bar: 5 cm). (G) HighE had fiber diameters of $0.859 \pm 0.311 \mu\text{m}$ and were relatively aligned (0.551 ± 0.106). (H) LowE⁺ fibers were smaller in size, though still significantly larger than HighE fibers ($0.865 \pm 0.164 \mu\text{m}$, $p > 0.01$), and maintained the same approximate alignment as the original LowE fibers (0.397 ± 0.076 , $p < 0.01$). (Scale bars: A, E 2 mm; Inset 500 μm ; C, D, G, H, 10 μm).

phase of the HighE evolved along the length of the electrodes (as described above), and therefore was exposed to more of the electrode area, the initial hypothesis was that the HighE fibers were subject to more overall electrostatic force during their flight time compared to the more localized LowE fibers, such that the HighE jet is preferentially attracted while LowE fibers are deflected to create the observed spatial bias.

To test this hypothesis, the effective force experienced by the LowE fibers was increased by adding 0.05 mg ml^{-1} of salt (NaCl) to the LowE polymer solution (referred to hereafter as LowE⁺) [30]. The LowE⁺ solution was confirmed to have increased conductivity (supplementary table S2) and the increased surface charge of the LowE⁺ fibers was evident from a much shorter stable jet region (supplementary figure 2(F)); this follows previous reports, where the addition of surface charge hastens the onset of the chaotic whipping phase [36]. With an increase in electrostatic force and with each polymer now having roughly equal stable jet length, the end result was a more centered distribution of HighE and LowE⁺ fibers (figure 2(E)). The LowE⁺ fibers were also smaller, consistent with a previous report on the effect of salt [37], a no change in the degree of alignment between the salt and salt-free condition was observed.

Additional experiments confirmed the role of electrostatic force versus the possible influence of stable jet length. By substituting the LowE solution with the

lower viscosity LowE_{mv} solution, the initiation of the whipping phase was now earlier compared to the HighE solution (supplementary figures S5(E), (F)); according to Hohman *et al* [38], the onset of whipping occurs once the polymer jet reaches a critical radius, with less viscous solutions reaching this stage earlier in the fluidic jet evolution. This still resulted in a biased fiber pattern, with HighE remaining dominant on the target substrate (supplementary figure S5(A)). Examining the later stages of jet evolution revealed that HighE fibers experienced a final pull towards the target area (supplementary figure S5(K)), further supporting the hypothesis that the elongated whipping profile of the HighE fibers resulted in greater electrostatic attraction. Adding salt to the LowE_{mv} solution (hereafter LowE_{mv}⁺), evidence of increased surface charge was again observed by a much shorter stable jet length (approximately 1.5 cm) and a more centered distribution of tandem fiber deposition with a defined region of overlap (supplementary figure S7). The resulting LowE_{mv}⁺ fibers were now equal in diameter ($0.774 \pm 0.287 \mu\text{m}$) to the tandem-spun HighE fibers ($0.859 \pm 0.311 \mu\text{m}$, $p < 0.01$) but maintained a reduced degree of alignment (0.247 ± 0.0586 , scaled from 0-random to 1-aligned). In summary, tuning polymer solutions used for heterogeneous T-ESP makes possible fiber constructs with both distinct fiber alignments and tailored distribution patterns.

Figure 3. Triple ESP and Divergent Tandem ESP. (A) An example of focused aligned electrospinning with 3 separate HighE solutions simultaneously (scale bar: 2 mm). A different dye was added to each solution (see Methods section). The blue autofluorescence of HighE was enhanced by adding Pyrene, though the residual blue autofluorescence of the other fiber populations modified the typical green and red dye colors to mint and pink, respectively. (B) The result of tandem ESP on an 'epsilon' gap electrode arrangement (shown in C), producing a region of overlapping fibers (scale bars, B: 2mm; D: 500 μm) which then diverge. Further details of the needle and target collector arrangements for these patterns are described in supplementary material figure S9. Fiber diameter is $0.788 \pm 0.279 \mu\text{m}$, consistent with previous HighE ESP fibers.

Robust tandem deposition and complex T-ESP fiber patterning

Once the optimization of T-ESP was established, the resulting tandem deposition was observed to be highly robust. Scaffolds were produced one at a time, taking approximately 5 min per scaffolds and an estimated 100% success rate. Given the robust nature of this approach, throughput could potentially be increased via deposition onto a narrow rotating disc collector [39], though care should be taken to avoid extraneous grounding points which may disrupt the electric focusing effect observed here.

Given the relative stability of this process, we extended this strategy to include multi-jet spinning to create different patterns of fiber deposition. For example, moving from two to three jets resulted in the simultaneous overlapping deposition of three populations of aligned fibers (figure 3(A)). When optimizing this process, achieving uninterrupted jet stability for all three polymers required an inter-needle separation of 5 cm, for a distance of 10 cm between the two outer needles. The outer polymer jets still converged onto the narrow gap electrode. However, this inter-needle spacing was an intrinsic limit to our system; farther separation resulted in fiber deposition on extraneous grounding points located within the electrospinning chamber. With a complete redesign of our current electrospinning apparatus, one could expect successful T-ESP processing with farther needle separations.

The target electrode arrangement was further altered to achieve a divergent pattern of deposition (figure 3(C); supplementary figure S9 for electrode details). Figure 3(B) shows two fiber populations which have both deposited onto a 'shared' bottom electrode in an aligned, overlapping manner (figure 3(D)). These fibers then diverged and separated, with the left polymer fibers (red) oriented

towards the left upper electrode and the right polymer fibers (green) directed towards the right electrode. This creates a unique fiber pattern with a degree of complexity not yet seen for ESP. All patterns described could also be replicated by performed T-ESP with a 5% solution of PEO in dH₂O, confirming that this process is compatible with other polymers and solvent (figure supplementary S8).

T-ESP applications

This new fabrication approach has an immediate application in the field of tissue engineering and regenerative medicine. The design of fibrous scaffolds follows a common biomimetic principle, with the intent of achieving an appropriate cell response by imitating the natural fibrous extracellular matrix found in the body. Current ESP scaffold can be tailored to elicit specific cell behavior, but fibers are typically modified homogeneously. However, this does not reflect the heterogeneous cell populations found *in vivo*. The possibility to create tissue scaffolds with purposely-designed heterogeneity may enhance scaffold effectiveness, exacting spatial control of cell response imparted by the properties of respective fiber types. Furthermore, bifurcated branching structures are ubiquitous throughout the body, including, but not limited to, nerves, vasculature, pulmonary and breast tissue [40–43]. The ability to make fibrous scaffolds as described in this work provides a promising tool for studying these types of biological systems, such as evaluating cellular decision-making processes at an ECM-like bifurcation, as well as for the development of tissue engineering solutions.

To show the utility of T-ESP fiber tissue scaffolds for the study of presenting a biological system with a defined decision point, we examined the impact of a heterogeneous divergent scaffold of HighE fibers and

Figure 4. Spatially modulated neurite outgrowth on a divergent heterogeneous Tandem ESP scaffold. An explanted DRG (large, round structure) grown on aligned fiber scaffolds of either HighE-Collagen (A) or HighE-Only (B) do not exhibit such a dramatic difference in neurite length, with measurements of $2232 \pm 74 \mu\text{m}$ and $2953 \pm 88.7 \mu\text{m}$ respectively. In contrast, a divergent T-ESP pattern (C) exhibits a distinctive difference between the two fiber types, comprised of HighE-collagen fibers diverging to the left half labeled, 'HighE-Collagen', and HighE fibers diverging to the right half labeled 'HighE-Only'. With a DRG explant placed at the bifurcation of the two fiber types, the resulting growth pattern shows a clear preference for the 'HighE-Collagen' domain with an average neurite length of $3632 \pm 217.4 \mu\text{m}$ versus the 'HighE-only' with a length of $1522 \pm 77.3 \mu\text{m}$ (scale bar 1 mm). The divergent pattern lends itself to automated Sholl Analysis (D), whereby the evaluation of the 10 longest neurites (E) can quickly be determined to examine the impact of creating a clear decision point within a fibrous scaffold.

an HighE-collagen blended fibers on neurite outgrowth (figure 4). An earlier study showed modulated neurite growth on ESP scaffolds of PCL/collagen blended fibers compared to scaffolds of PCL-only fibers [44]. For our particular polymer system, an explanted dorsal root ganglion (DRG) grown on aligned fiber substrates that were homogeneously HighE-collagen or HighE-only fibers exhibited comparable levels of neurite growth, with a neurite length of $2232 \pm 74 \mu\text{m}$ and $2953 \pm 88.7 \mu\text{m}$, respectively (figures 4(A), (B)). This suggests that for the HighE polymer, the addition of collagen has limited impact on neurite growth.

In contrast, our divergent scaffold resulted in notably longer neurite extension on the HighE-collagen fibers compared to HighE-only fibres (figure 4(C)). Neurites also followed the fiber orientation, creating a divergent growth pattern that facilitated rapid assessment of the competitive outgrowth via automated Sholl Analysis (figure 4(D), supplementary figure S13). The plotted length of maximum neurite growth for each condition (figure 4(E)) clearly shows that the HighE-collagen portion of the

divergent scaffold achieved a neurite length of $3632 \pm 217.4 \mu\text{m}$ while the HighE-only fibers supported approximately half the amount of growth, with a length of $1522 \pm 77.3 \mu\text{m}$. In contrast, a divergent scaffold consisting of HighE-only fibers exhibits no visible bias (supplementary figure S14), indicating that it is the combination of the divergent fiber pattern and the organized fiber heterogeneity produced by the T-ESP process that stimulates this defined growth pattern. Overall, this suggests that neurites will readily grow on oriented nanofibrous scaffolds even in the absence of a preferred option. But if given a choice, a clear preference can be determined. This presents unique biofabrication possibilities for neural tissue engineering, such as generation of a nanofibrous scaffold for the spatially selective promotion of different neural subpopulations or a general tissue engineering approach for sorting of a heterogeneous cell population.

A gradient of fiber alignment could also spatially modulate cell morphology and, thus, creating a gradient of cell function. The spatial control provided by the T-ESP process also provides a means to investigate

the fabrication tolerances required in order to elicit such a graded response. Figure S10 shows the modulation of Schwann cell morphology on a T-ESP scaffold of aligned HighE fibers and less aligned LowE_{mv}⁺ fibers, where such differences in Schwann cell morphology are known to modulate their production of neurotrophic factors [45].

The application of this technology is not limited to the field of Tissue Engineering. Initial trials have also employed tandem scaffolds to separate mixed solutions on the basis of fiber affinity (supplementary figure S11), showing promise in the field of Separation Technologies. This fabrication technique can be used to prepare heterogeneous catalytic or filtration substrates or high surface area nanofibrous cathode/anode electrode configurations able to sequester specific analytes.

Conclusions

Until now, the ease and promising characteristics of ESP has found limited applicability due to limitations in the ability to control and customize the resulting fiber meshes. This work presents the highly accessible T-ESP approach, providing the versatility to create an array of micro- and nano-scale fibrous constructs with complex, ordered patterns. At the same time, this work provides general considerations for optimal polymer selection as well as furthering the understanding of the ESP process. Though a promising tool for tissue engineering applications, this approach holds potential for other fields. The methodology presented here further extends the growing arsenal of ESP solutions to create increasingly complex polymer fiber constructs in a simple manner to address the growing demands of new material and engineering challenges.

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Conflict of Interest

Authors declare no conflicts of interest.

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