HHS Public Access

Author manuscript

Angew Chem Int Ed Engl. Author manuscript; available in PMC 2020 December 24.

Published in final edited form as:

Angew Chem Int Ed Engl. 2019 March 18; 58(12): 3948–3951. doi:10.1002/anie.201814474.

Promoting the Outgrowth of Neurites on Electrospun Microfibers by Functionalization with Electrosprayed Microparticles of Fatty Acids

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Abstract

Controlling the outgrowth of neurites is important for enhancing the repair of injured nerves and understanding the development of nervous systems. Herein we report a simple strategy for enhancing the outgrowth of neurites through a unique integration of topographical guidance and a chemical cue. We use electrospray to easily functionalize the surface of a substrate with microparticles of natural fatty acids at a controllable density. Through a synergistic effect from the surface roughness arising from the microparticles and the chemical cue offered by the fatty acids, the outgrowth of neurites from PC12 cells is greatly enhanced. We also functionalize the surfaces of uniaxially aligned, electrospun microfibers with the microparticles and further demonstrate that the substrates can guide and enhance directional outgrowth of neurites from both PC12 multicellular spheroids and chick embryonic dorsal root ganglia bodies.

Keywords

electrospun fibers; fatty acids; microparticles; neurite outgrowth; topographic cues

Controlling the outgrowth of neurites is of great importance for enhancing the repair of injured nerves.[1–3] The neuron utilizes the so-called growth cone, a highly motile structure at the end of the neurite, to sense the surrounding environment. The growth cone can be approximately 10–760 μm^2 in area depending on the type of the neuron and the extracellular matrix.[4–8] Through interactions with the surroundings, including the topographic features

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Conflict of interest

The authors declare no conflict of interest.

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201814474.

on a substrate[9–12] and the diffusible guidance molecules in a medium,[13,14] neuritis can undertake different path-finding behaviors. The correlation between the neuronal behavior and the topographic cue on a substrate was initially investigated using lithographically fabricated structures[15] and electrospun aligned fibers.[16] In parallel, substrates decorated with Au[11,17] or Ag nanoparticles,[18] anodized aluminum oxide membranes,[19] silica beads,[8,20] and electrosprayed poly(lactic-*co*-glycolic acid) (PLGA) microparticles,[21] have all been shown to be able to drastically affect neuronal responses. The optimization of the topographical features holds the key to achieve the desired neural behaviors. In order to stimulate neurons in a controllable and systematic manner, it is necessary to fabricate substrates with reproducible and rigid topographical features. To this end, electrospray offers a simple and effective method for producing and depositing particles on a surface to construct substrates with controllable topographical features by simply varying the density of the particles.[21]

Topographical cues often act in concert with biochemical cues, working synergistically to control the neural behaviors. The outgrowth of neurites from the cell body requires the generation of an additional plasma membrane, involving the activation of phospholipases to produce soluble fatty acids.[22,23] It has been demonstrated that medium-chain saturated fatty acids (e.g., octanoic, nonanoic, decanoic, and lauric acids) can enhance never growth factor (NGF)-induced neurite outgrowth from PC12 cells by activating the mitogen-activated protein kinases.[24] In a completely different application, fatty acids have been explored as a new class of gating materials for controlled release by leveraging their well-defined, sharp melting points.[25–29] In principle, solid microparticles of fatty acids can be used as a multi-functional platform to present topographic guidance while offering a chemical cue and serving as a gating material for controlled release.

Herein, we utilized electrospray to fabricate solid microparticles from a mixture of lauric acid and stearic acid, which was formulated with a sharp melting point at 39°C to ensure structural integrity when cultured with cells.[25–27] We varied the densities of the microparticles on glass slides to construct substrates with controllable topographical cues to optimize the outgrowth of neurites from PC12 cells. We also examined the influences of the microparticles on neurite outgrowth by benchmarking against free fatty acids at different concentrations. Furthermore, we deposited the microparticles on uniaxially aligned, electrospun microfibers at an optimal density to direct the outgrowth of neurites from both PC12 multicellular spheroids and chick embryonic dorsal root ganglia (DRG) bodies.

First, we performed electrospraying to fabricate microparticles of fatty acids. Figure S1, A and B in the Supporting Information, shows scanning electron microscopy (SEM) images of the as-deposited particles, indicating that they were more or less spherical in shape, together with a rough surface. The average diameter of the particles was 4.36 ± 0.33 μm (Figure S2). Since the microparticles are deposited randomly on the glass slide, we could easily tailor the densities of the particles on the glass slides by changing the collection time to construct substrates with different degrees of surface roughness.[21] Figure S1, C–F, shows SEM images of the particles on glass slides collected for 1, 2, 5, and 8 min. The extension of collection time resulted in an increase in density for the microparticles and thus an increase in average surface roughness for the substrate.

We then investigated the influence of the deposited microparticles on the outgrowth of neurites from PC12 cells. The substrates did not cause any major cytotoxicity until a collection time of 8 min for the particles (Figure S3). From Figure S4A, neurites were extended in a random orientation. Both the average and the longest lengths of the neurites increased when the surface roughness of the substrate increased from low (with a collection time of 1 or 2 min for the microparticles) to moderate (with a collection time of 5 min) and subsequently decreased when the surface roughness was further increased from moderate to high (with a collection time of 8 min; Figure S4B). On the substrate with a collection time of 5 min, the average length of the neurites was the greatest (154.4 ± 32.7 μm), which was about six-times longer than that of cells cultured on the glass slide without microparticles, and the largest number of neurites were extended from cells (Figure S4C). As shown in Figure S5, the cells cultured on the substrate with moderate density of particles expressed the highest levels of three types of genes related to neurite outgrowth (growth associated protein 43, GAP43; neurofilament heavy, NEFH; and tubulin beta-3, TUBB3) among all groups. Thus, the density of the microparticles, that is, the topographic feature on the substrate, had a major impact on the outgrowth of neurites. The size polydispersity of the electrosprayed particles should not cause significant changes to the neurite outgrowth.

To understand the modulation of neural behaviors by the density of the microparticles, we investigated how the particles dispersed in a culture medium affected the neurite outgrowth. The microparticles had no cytotoxicity at concentrations of 10 and 50 μg mL^{-1} (Figure S6A). At a concentration of 100 μg mL^{-1}, the cell viability was decreased. From Figures S6, B and C, as the concentration of the microparticles was increased, both the average and the longest lengths of the neurites were increased, indicating that the dispersed microparticles could still promote neurite outgrowth. Since lauric acid and stearic acid can be slightly dissolved into the medium, we hypothesized that the promotion of neurite outgrowth could be attributed to the dissolved free fatty acids. In this regard, we studied the influence of free fatty acids (a mixture made of lauric acid and stearic acid at a mass ratio of 4:1) at different concentrations on the outgrowth of neurites. As shown in Figure S7A, free fatty acids showed cytotoxicity until at concentrations of 50 and 100 μg mL^{-1}. With regard to the neurite outgrowth, the cells incubated in the medium containing 10 μg mL^{-1} of free fatty acids projected significantly longer neurites (58.7 ± 4.0 μm) than those incubated in the media with 1 μg mL^{-1} of free fatty acids ($P < 0.01$) and without free fatty acids ($P < 0.01$) (Figure S7, B and C). When the free fatty acids were used at 50 μg mL^{-1}, the average length of the neurites was 54.2 ± 3.1 μm, showing no significant difference from the group with the free fatty acids at 10 μg mL^{-1}.

Our results show that the cells incubated in the medium supplemented with 10 μg mL^{-1} of free fatty acids projected longer neurites than those in the media supplemented with 50 and 100 μg mL^{-1} microparticles. This observation also indicates that the effect provided by the chemical cue of the soluble fatty acids was rather limited as the neurites could only be increased by about two times in terms of length. In comparison, when the microparticles were deposited on glass slides, the length of the neurites could be enhanced by almost six times. Taken together, it is clear that the surface roughness of the substrate also greatly contributed to the outgrowth of neurites. The neurites extending from PC12 cells that were cultured on substrates with moderate roughness were notably longer than those on substrates

with both low and high surface roughness. These results agree with a previous report in that the moderate roughness of a substrate constructed from electrosprayed PLGA microparticles resulted in the longest neurite outgrowth from DRG bodies.[21] On a substrate, the neurites utilize growth cones to guide their pathways, and the adjacent surface features may work as points of adhesion for the growth cones. On the substrate with moderate surface roughness, the neurites use the microparticles as anchoring points to further support the growth cones for moving and finding pathways, resulting in elongation with little structural disruption for the extending neurites. In comparison, on the substrate with a high density of microparticles, there are many available points of adhesion, resulting in short and multi-directional turns for the neurites in order to navigate across the tortuous surface. Besides, a high density of microparticles could cause cytotoxicity to the cells, thus inhibiting the outgrowth of neurites.

For repairing injured nerves, it is important to guide the outgrowth of neurites along one direction. Uniaxially aligned fibers fabricated by electrospinning have been widely used to guide the outgrowth of neurites along the fiber alignment.[30–32] Herein we utilized aligned poly-(*ε*-caprolactone) (PCL) microfibers to collect the microparticles for 1, 2, 5, and 8 min, respectively (Figure 1). The average diameter of the fibers was 987 ± 72 nm, and the fiber density was approximately 336 fibers per mm (or with an average separation of 1989 nm between adjacent fibers). The electrosprayed particles could adhere strongly to the fibers or glass slide as a result of the presence of residual solvent. The particles tended to be localized both on the fiber and between the fibers, and the percentages of particles located at different positions did not vary with the collection time.

To better demonstrate the directional outgrowth of neurites, we cultured PC12 multicellular spheroids with a diameter of about 200–400 μm on the different substrates. From Figure S8, the substrates did not cause any major cytotoxicity until a collection time of 8 min for the particles. From the fluorescence micrographs in Figure 2A and Figure S9, in the presence of uniaxially aligned fibers, the neurites were guided to extend along the fiber alignment. As shown in Figure 2B, in comparison with those on the bare fibers, longer neurites were extended from the multicellular spheroids for the substrates comprising of aligned fibers and decorated with the microparticles. On the substrates with a collection time of 5 min, the average length of the neurites was the longest (430.6 ± 22.9 μm). The neurites were extended along the fibers and across the particles. The aligned fibers guided the directional outgrowth of the neurites while the particles provided anchoring points to further support the growth cones for finding pathways. Since the growth cone at the tip of a growing neurite can cover a relatively wide angle, the exact locations of the particles relative to the fibers should not have a major impact on the extension of neurites. Regardless of the substrates utilized to collect the microparticles, glass slides or aligned fibers, their topographic features showed a similar impact on the outgrowth of neurites, with the best performance corresponding to a moderate density of microparticles. Meanwhile, the fatty acids offered the chemical cue to accelerate the neurite outgrowth. Furthermore, the multicellular spheroids on the substrates with a collection time of 5 min expressed the highest levels of genes related to neurite outgrowth among all groups (Figure 2C). The influence of the substrates on the expression levels of genes showed the same correlation to the length of the neurites.

We also utilized chick embryonic DRG as another neuron model to investigate the influence of the different substrates on neurite outgrowth. As shown in Figure 3, the neurites extending from the DRG cultured on the different substrates were parallel to the alignment direction of the fibers. From Figure 4, on the bare fibers, the average length of the neurites was 869.6 ± 19.1 μm. On the aligned fibers deposited with microparticles for 2 min, both the average and the longest lengths of the neurites were slightly increased ($P < 0.05$). By further increasing the collection time to 5 min, the average length of the neurites was significantly increased to 1202.3 ± 29.7 μm ($P < 0.01$). Taken together, the electrospun fibers functionalized with microparticles of fatty acids could also promote the outgrowth of neurites from DRG bodies.

In summary, we have fabricated microparticles of fatty acids by electrospray and varied their density on a collector to construct a substrate with the optimal topographical features to promote neurite outgrowth. On the substrates constructed by varying the densities of microparticles deposited on glass slides, PC12 cells showed sensitivity to the topographical features. By functionalizing uniaxially aligned, electrospun microfibers with a moderate density of the microparticles, we could maximally guide the directional outgrowth of neurites from both PC12 multicellular spheroids and DRG bodies. The neurite outgrowth could be attributed to the surface roughness of the substrates and the chemical cue offered by the fatty acids. The fiber density together with the surface coating can also be varied to control the growth direction of the neurites.[30] For translational use, the uniaxially aligned fibers can be placed a on non-woven mat of random fibers prior to electrospray of microparticles. The bi-layer structure can then be rolled-up to form a conduit for peripheral nerve repair.[32] In addition, known for their reversible solid–liquid phase transition in response to temperature variation, fatty acids have been used as a class of thermosensitive materials for the controlled release of various bioactive agents.[25–29] This unique capability can be combined with various types of growth factors to further enhance neurite outgrowth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by a grant from the National Institutes of Health (R01 EB020050) and startup funds from the Georgia Institute of Technology.

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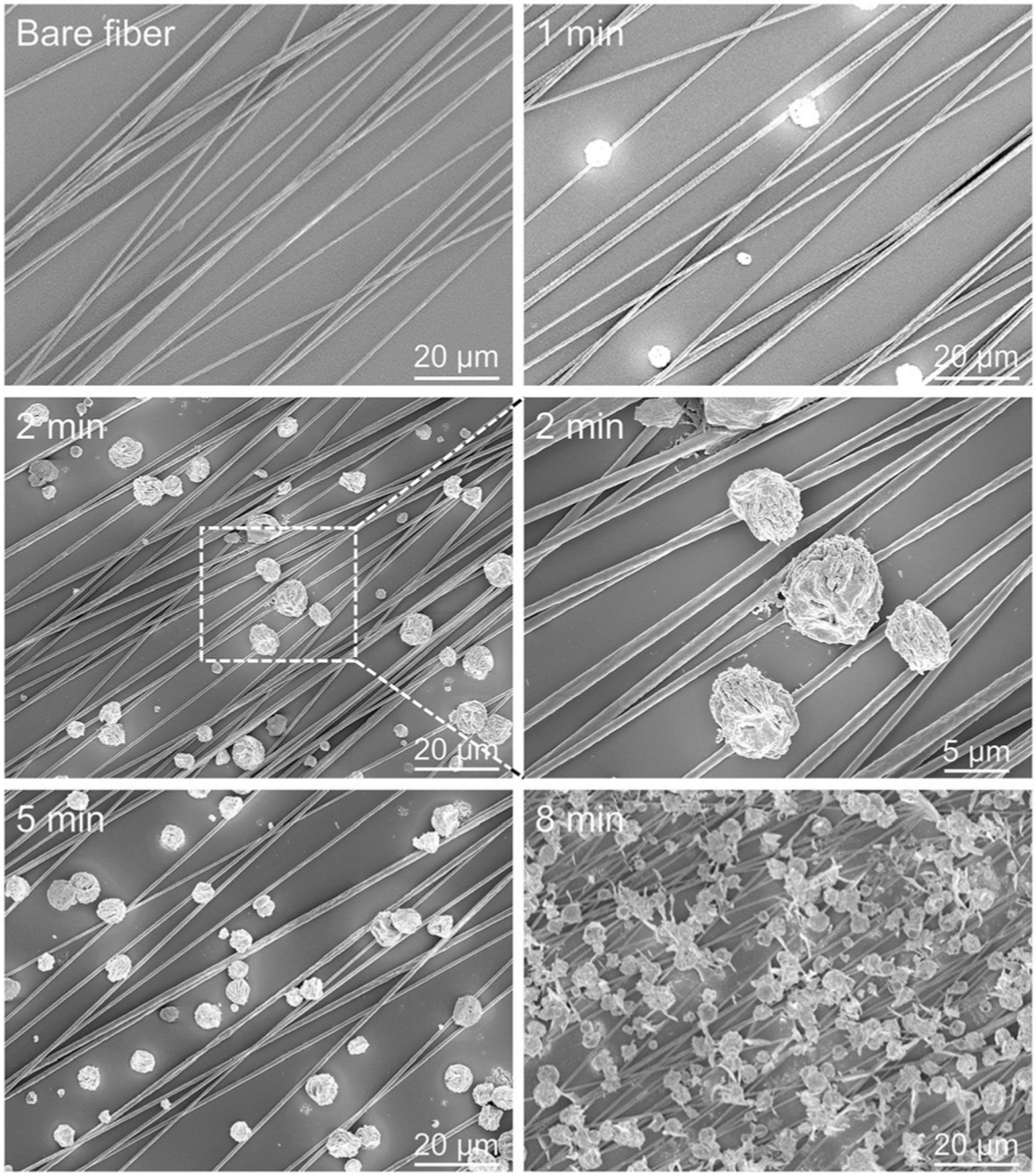

Figure 1.

SEM images of electrosprayed microparticles collected on uniaxially aligned, electrospun PCL fibers for 0, 1, 2, 5, and 8 min, respectively.

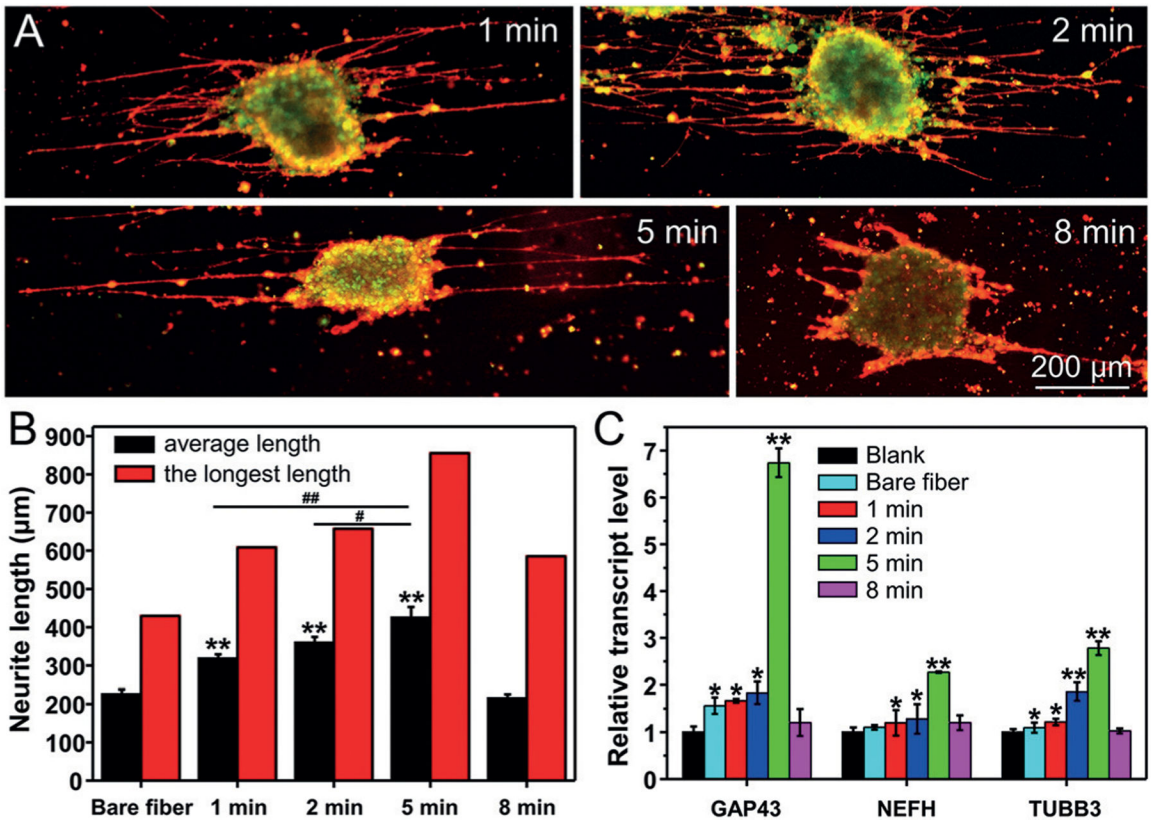

Figure 2.

A) Fluorescence micrographs of PC12 multicellular spheroids after culture for six days on the uniaxially aligned, electrospun fibers deposited with microparticles for 1, 2, 5, and 8 min, respectively. The PC12 multicellular spheroids were stained using Neurite Outgrowth Staining Kit. The yellow color corresponds to an overlay of the red and green fluorescence from the cell membrane and cell viability indicators, respectively. B) The average and the longest lengths of neurites extending from PC12 multicellular spheroids cultured on the different substrates. $^{**}P<0.01$ when compared to that in the group of bare fiber. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ when compared those with the group of fibers deposited with microparticles for 5 min. C) The relative transcript levels of genes expressed in PC12 multicellular spheroids cultured on the different substrates. $^{*}P<0.05$ and $^{**}P<0.01$ with reference to that in the blank group.

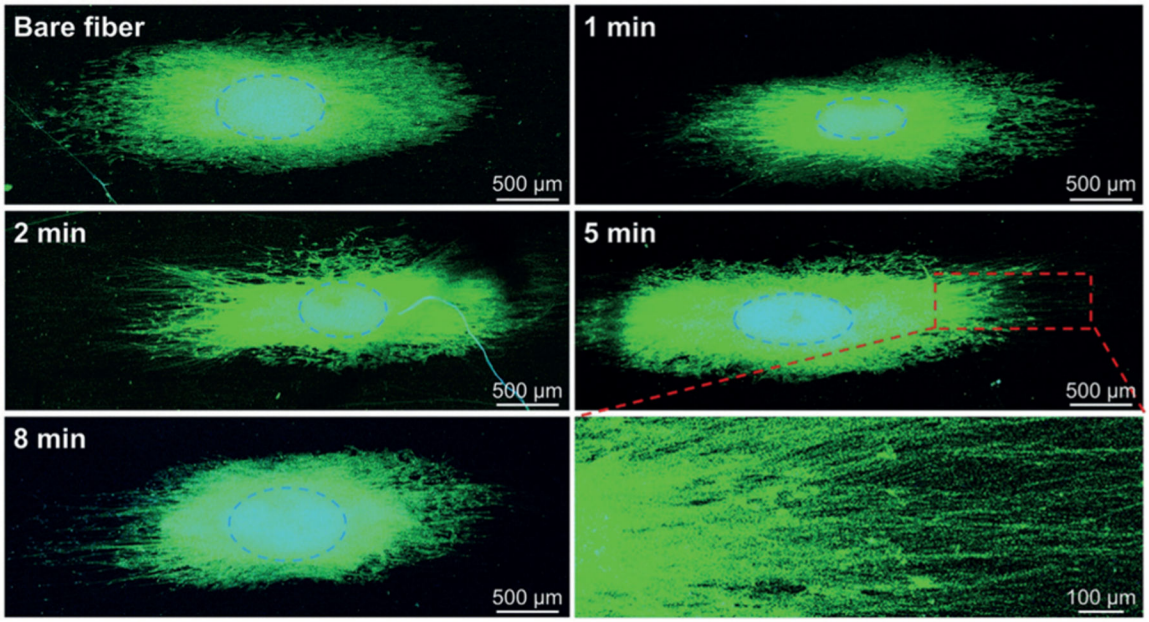

Figure 3.

Fluorescence micrographs of the typical neurites extending from chick embryonic DRG cultured on the uniaxially aligned, electrospun fibers deposited with microparticles for 1, 2, 5, and 8 min. The neurites were immunostained with Tuj1 marker (green), and the nuclei were stained with 4',6-diamidino-2-phenylindole (blue). The boundary of the DRG body is indicated with a broken ellipse.

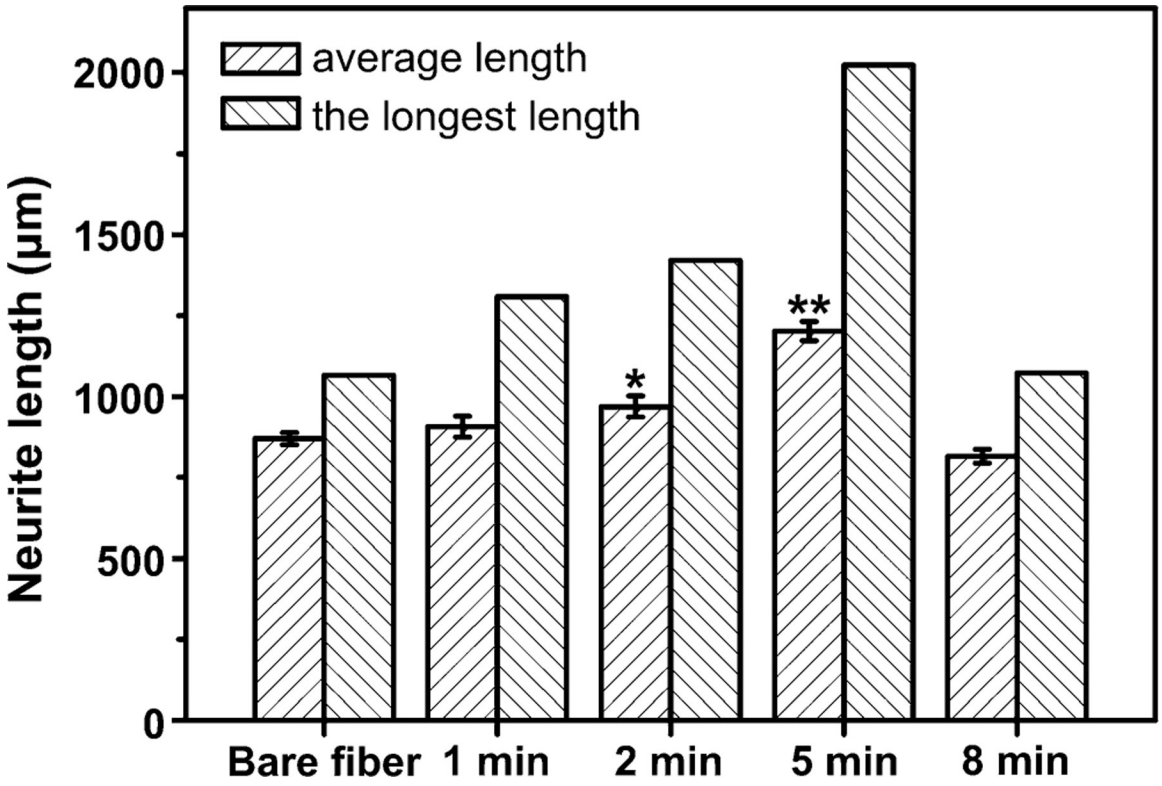

Figure 4.

The average and the longest lengths of the neurites extending from DRG cultured on the uniaxially aligned fibers deposited with microparticles for 1, 2, 5, and 8 min, respectively, as measured from the fluorescence micrographs. **P*<0.05 and ***P*<0.01 when compared to that in the group of fibers without microparticles (bare fiber).