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Enhanced Neurite Outgrowth on Electrically Conductive Carbon Aerogel Substrates in the Presence of an External Electric Field

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COMMUNICATION

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Previous works from our laboratory have firmly established that aerogels are a suitable substrate to elicit accelerated neurite extension. On non-conducting aerogels, in the presence of an externally-applied DC bias, neurons extended neurites which were preferentially aligned towards the anode. In this investigation, we sought to determine whether electrically-conductive carbon aerogels elicited a more robust alignment of neurites toward the anode than non-conductive aerogels due to the capacity of conductive aerogels to sustain a current, thereby providing a direct interface between neurons and the external electrical stimulus. To determine if this was the case, we plated PC12 neuronal cells on electrically conductive carbon aerolges derived from acetic acid-catalized resorcinol formaldehyde aerogels (ARF-CA) and subjected them to an external electric field. The voltages applied at the electrodes of the custom-built electro-stimulation chamber were 0 V, 15 V, and 30 V. For each voltage, the directionality and length of the neurites extended by PC12 cells were determined and compared to those observed when PC12 cells were plated on non-conductive aerogels subjected to the same voltage. The results show that the directionality of neurite extension was similar between conductive and non-conductive aerogels. A higher neurite length difference was observed on conductive aerogels with increasing voltage, 43% and 106% for 0-15 V and 0-30V respectively, compared to non-conductive aerogels, 12% and 20%. These findings indicate that conductive carbon aerogels have a greater potential as scaffolds for nerve regeneration than non-conductive ones.

Introduction

Using external cues to influence the length and orientation of neurites during nerve generation provides a unique opportunity to maximize the efficiency of prosthetic systems for nerve regeneration. To achieve this goal, a large body of research has been conducted[1-17] and is still ongoing. The two main influences that have been studied are (1) substrate-related properties e.g., stiffness and topography[2,4,18-19] and (2) electrical stimulation involving alternating current (AC) or direct current (DC) exogenous fields[3,20-23]. In earlier studies we have demonstrated that aerogels provide a unique combination of customizable properties that enhance neuronal cell responses advantageous to nerve regeneration[1-8,17-18]. Compared to other types of aerogels, carbon-based aerogels have the advantage of being electrically conductive and can be customized to have pores with a diameter in the µm to nm range[25-27]. The pore structure of aerogels heavily influences the surface roughness (Ra) and we have shown previously that Ra plays a key role in neurite outgrowth and alignment[18].

In this study, we examine the effect of an externally applied DC bias on the orientation and length of neurites extended by PC12 neuronal cells plated on ARF-CA. Past investigation showed that pristine ARF-CA (pore diameter > 1 µm) and carbon aerogel derived from base-catalyzed resorcinol formaldehyde aerogels (BRF-CA) (pore diameter < 1 µm) substrates are not toxic to PC12 cells and that, when these aerogels are coated with a collagen layer, they enable cell attachment and subsequent neurite outgrowth[4]. The distinctly different pore structure of ARF-CA and BRF-CA leads to different surface roughness (Ra) values. In our previous investigation, we compared key neuronal cell responses to ARF-CA and BRF-CA and found differences which could be attributed in the aerogel's Ra[4]. Namely, the length of neurites extended by PC12 neuronal cells plated on ARF-CA was less than that observed for cells plated on BRF-CA. Because of this finding, we chose ARF-CA for this study in which we evaluate the effect of 0 V, 15 V, and 30 V externally applied DC bias on the length and directionality of neurites extended by PC12 cells. Results were compared to those of an earlier study from our laboratory[3] in which we developed an electro-stimulation chamber to study neurite extension by PC12 cells plated on non-electrically conducting polyurea crosslinked silica aerogels (PCSA)[24] and exposed to the same range of voltage used here. Results from this current work show that in the presence of a current, PC12 cells plated on ARF-CA extend neurites that were much longer than that observed in previous studies[2-4,17-18] but that these neurites did not orient in a preferential direction. The significance of these results for

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the potential of ARF-CA to serve as scaffolds for nerve regeneration is discussed.

Experimental Section

Aerogel Synthesis

Carbon aerogel monoliths were prepared as follows: first, resorcinol (1.23 g, 0.0112 mol) and 37 % formaldehyde solution (1.79 g, 0.0224 mol) were dissolved in water (1.5 ml), followed by the addition of 0.44 µl of acetic acid. The reaction mixture was then transferred to glass molds and cured at 80°C for at least 12 h. The resulting organic hydrogels were washed with acetone to remove the water and then dried with supercritical CO_2. These organic aerogels were carbonized at 1050°C for 3 h under nitrogen, yielding final carbon aerogels with densities ~0.5 g/cm^3, as determined by measurement of volume and mass. Aerogels were segmented with a razor blade into 5 mm × 5 mm squares with a 2 mm thickness prior to cell culture steps. Electrical conductivity of the aerogels was determined by the two-probe method. Measurements yielded a conductivity of 10 S/cm[26-28]. To measure the magnitude of current supported by these aerogels a Keithley Instruments 2400 power supply was used to provide 3 V, 15 V and finally 30 V to the aerogels. The source voltage was set, and the current was measured across the two probes. At 3 V the measured current was ~ 100 mA while at 15 V and 30 V the maximum current of 1 A was reached. Table 1 summarizes the properties of ARF-CA.

Electro-stimulation chamber

A custom-built electro-stimulation chamber was constructed to provide a constant electrical bias during cell culture. The assembly was constructed from copper plates, 30 AWG wires (American Wire Gauge), and 3.5 cm tissue culture polystyrene (TCPS) dishes. Two wires were attached to each copper plate to provide electrical connectivity. The electrodes (copper plates) were insulated with a 1 mm layer of Sylgard 184 (Dow Silicones Corporation, Midland, MI, USA) and attached to the lid of a 3.5 cm TCPS. To prevent electrolysis and consequent production of toxic byproducts the copper electrodes were fully encapsulated in a Sylgard 184 casing (Figure 1a) prior to immersion in cell culture medium. Figure 1b shows a schematic diagram of the different components of the electro-stimulation chamber full assembled (medium not shown). One end of the wires was fed through the TCPS using small holes while the other end was connected to a power supply. An ARF-CA substrate was glued to the bottom of the TCPS as shown in Figure 1. The device provided a sterile environment for cell culture while also exposing the cell-substrate system to an electric field. For the sake of consistency and comparative analysis, all design specifications of the device were the same as in a previous investigation with (PCSA)[3]. The insulating nature of Sylgard 184 reduces the actual voltage experienced by the cell-substrate system and was extrapolated computationally. Table 1 summarizes the parameters used to calculate the current magnitude in the ARF-CA substrate (also see Discussion section).

Table 1: Aerogel properties compared

	ARF-CA	PCSA
Porosity	77%	~70%
Conductivity	10 S/cm	N/A
Young's Modulus	1 GPa	3 MPa
Density	500 mg/cm^3	700 mg/cm^3
Pore Diameter	5 µm	~50 nm
Surface Roughness	1.15 µm	0.14 µm

Material Properties of ARF-CA and PCSA: Material properties (Conductivity, porosity, Young's modulus, density, pore diameter, and surface roughness) for ARF-CA and PCSA.

with type 1 collagen in a sterile biological hood. Sterilization was performed using a 100% ethanol wash and a 30 min exposure to 254 nm UV light[2-4,17-18]. Sterilized substrates were coated with 0.05 mg/ml rat type I collagen (Invitrogen, Carlsbad, CA, USA) in 20mM acetic acid to form a 4 µg/cm^2 layer of collagen. The collagen solution was placed on top of the substrate and allowed to settle for 1 h after which excess collagen was removed and the substrates were washed with serum-free RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA). PC12 neuronal cells, a cell line derived from a rat pheochromocytoma (a tumor derived from adrenal medulla neuroendocrine chromaffin cells), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured using RPMI 1640 complete medium supplemented with penicillin and streptomycin, 10% horse serum, Glutamax, HEPES buffer, and 5% fetal bovine serum. Cells were seeded at a density of 1x10^4 cells/cm^2 and cultured with RPMI medium containing 5 mg/ml nerve growth factor (NGF).

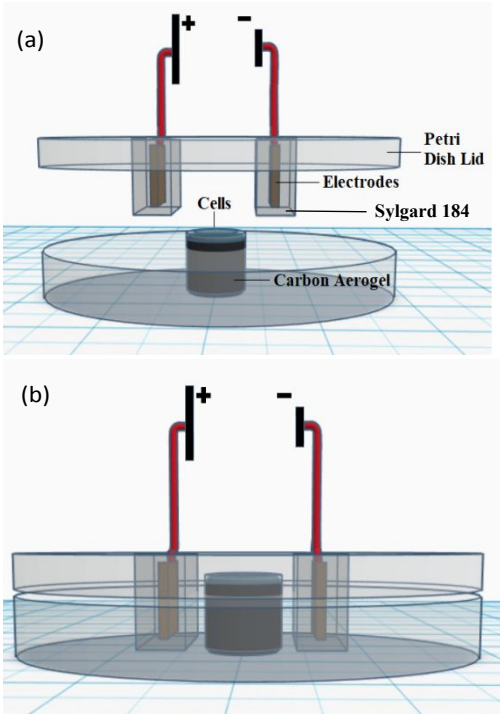

Figure 1: Schematic diagram of the custom-built electro-stimulation device used in this study to apply controlled exogenous electric fields (a) prior to and (b) post assembly. To prevent electrolysis copper electrodes were insulated from the surrounding cell medium using the dielectric Sylgard 184.

The cells were then placed in a 5% CO_2 tissue culture incubator at 37 °C. Two different voltages 15 V and 30 V were used for this study. Three independent trials (N = 3) were performed for each voltage and a control (ARF-CA without a DC bias) was run in parallel for each trial.

Before each experiment, PC12 cells were "primed" for neural differentiation by plating them on collagen 1 coated TCPS. Priming of cells consisted of culturing cells with NGF for 8 days with a complete medium as reported previously. This medium was replaced every 2-3 days. After 8 days, the cells were harvested and frozen in liquid nitrogen until used for the experiments[2-4, 17-18].

Assessment of Neurite Length and Orientation

Neurite length and orientation were measured on SEM (Scanning Electron Microscope) images using an open-source software from National Institutes of Health (NIH) Image J (version 1.53d). The scale bar of the SEM images was used to calibrate the measurement tools allowing for an accurate measurement of neurite length, as discussed in detail in prior publications[2-4,17-18]. A schematic diagram of how the neurite length and neurite orientation were defined is provided in supplementary content (Figure S1a). Branches arising from a neurite were not considered[2-4,17-18]. Neurite orientation was also evaluated using a straight-line tool; details have been provided in Figure S1b which shows a polar plot using the cell body of the PC12 cell as the origin and is consistent with metrics used in earlier studies[2-4,17-18].

Profilometry

The Profilm3D (Filmetrics Inc. San Diego, CA, USA) profilometer was used to obtain an overview of the surface of the aerogels before and after completing the cell culture steps. Samples were prepared in the same manner as for SEM imaging and the surface of each substrate was imaged in 200 µm x 200 µm intervals to obtain surface roughness of each aerogel type[17-18]. Table 1 shows the difference in surface roughness of both PCSA and ARF-CA. Figure 2a shows a high-resolution 3D image of the surface roughness of ARF-CA sample prior to cell culture while Figure 2b shows the surface of the aerogels in the presence of PC12 cells.

Statistical Analysis

Statistical analysis was performed using a two-tailed student's t-test which is intended for determining a p-value. For this investigation a $p < 0.05$ was considered as statistically significant[27]. Neurite length and neurite orientation were calculated for control (0 V) and other (15 V and 30 V) for three independent trials (N = 3). Each trial consisted of 200 measurements. The error bars (Figure 3a) represent the standard error of mean of the three independent trials (N = 3).

Results

Impact of DC Electrical Bias on Neurite Length

The effect of exogenous DC electrical bias on the extension of neurites by PC12 cells on electrically conducting carbon aerogels was evaluated (Figure 3a). Results indicate that increasing the magnitude of the applied bias leads to an increase in neurite length. A 15 V bias increased neurite length by 36% compared to 0 V. With a 30 V bias, neurite length increased by 36% and 70% compared to 15 and 0 V, respectively.

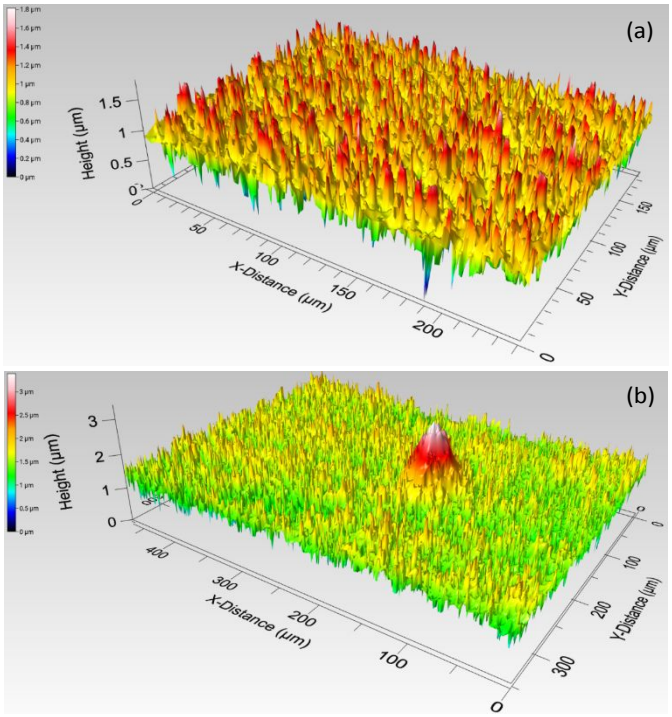

Figure 2: High-resolution 3D surface profilometry images of ARF-CA substrate (a) prior to and (b) post cell culture. Color scale bar corresponds to surface height information.

Comparison of these results to those obtained with PC12 cells plated on PCSA[2,3] subjected to an applied bias shows that conductive aerogels are much more efficient than PCSA in stimulating and sustaining neurite extension (Figure 3b). ARF-CA in the presence of 15 V applied bias showed an increased regeneration rate of 43% (compared to the control, 0 V). For the same substrate, in the presence of 30 V, neurites increased regeneration rate by 106% (compared to the control, 0 V). In the case of PCSA these experiments lead to only 12% and 20% in the presence of 15 V and 30 V respectively. The least remarkable increase in neurite regeneration rate was observed in TCPS which only increased by 6% and 15% in the presence of 15 V and 30 V applied external bias.

Impact of DC Electrical Bias on Neurite Orientation

The impact of exogenous DC fields on the orientation of neurites extended by PC12 cells was evaluated and results are shown in Figures 3c and 3d for applied bias of 15 V and 30 V, respectively. The data is presented as the percentage of neurites in each orientation in increments of 20° angles. PC12 cells grown on ARF-CA in the presence of 15 V or 30 V external DC bias did not show any preferred orientation and the direction of growth was independent of the anode/cathode location.

Furthermore, Figure 2 shows the surface roughness of the ARF-CA substrate before and after the process of cell-culture. The

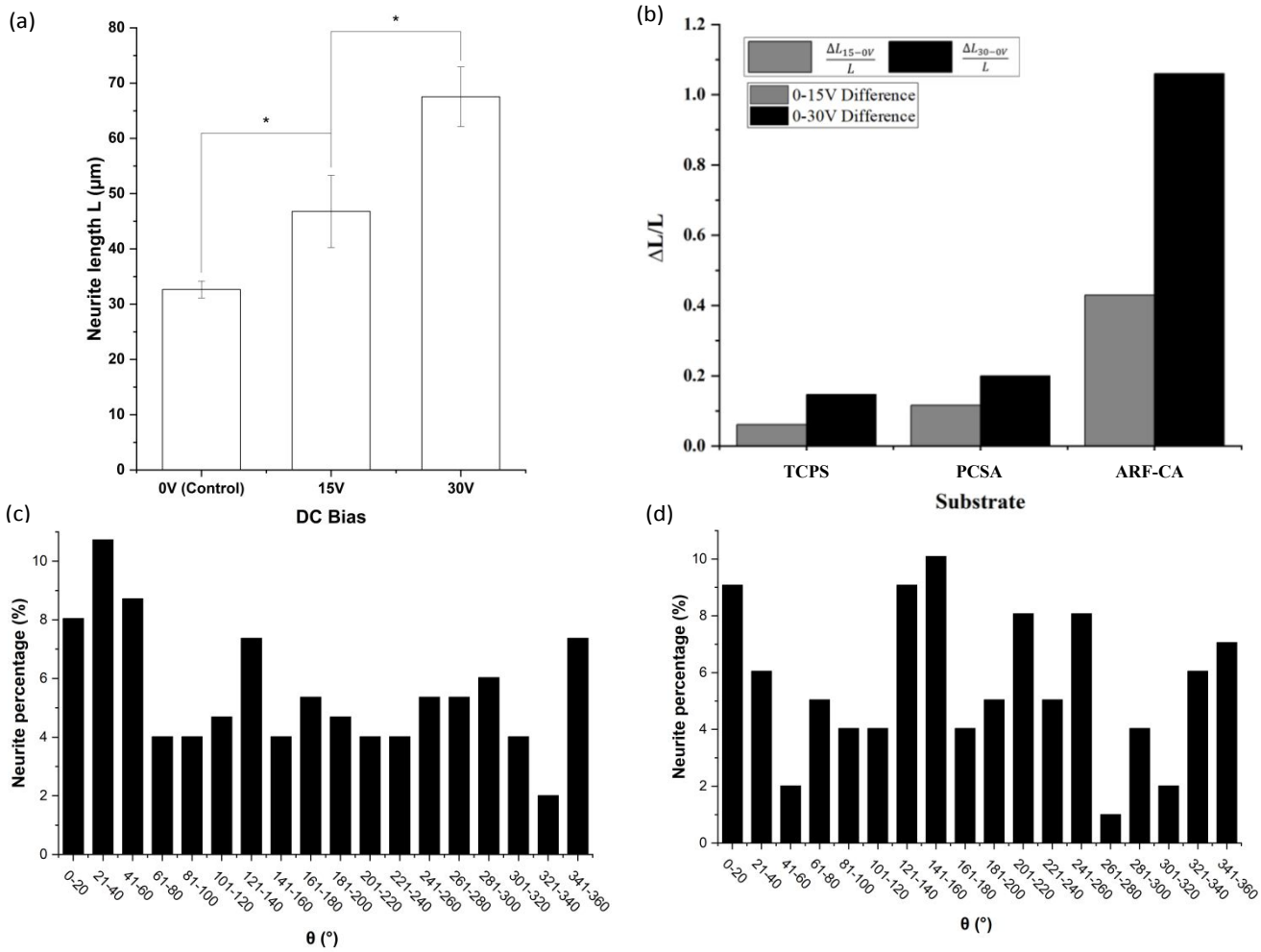

Figure 3: (a) Average neurite length as a function of increasing DC bias on ARF-CA substrate, for 3 independent trials (N = 3) each trial consisted of 200 independent measurements. Asterisks (*) denote values that are statistically significant ($p < 0.05$) with a p value of 0.027 and 0.011 between 0-15 V and 15-30 V respectively. (b) Normalized neurite length difference (ΔL) between 0-15 V and 0-30 V for three different substrates: TCPS, PCSA[3], and ARF-CA. (c) PC-12 cells neurite orientation (in 20° intervals) for (c) 15 V and (d) 30 V.

images show the lack of change in the substrate roughness when the cell culture step was performed. The overall initial ARF-CA roughness that was observed is transferred without any detectable alteration to the post processing stage of the study.

Discussion

PC12 cells plated on electrically conductive amorphous carbon aerogels in the presence of external DC bias extend neurites much longer that PC12 plated on other aerogel types

In our earlier studies we have shown that aerogels are conducive to enhanced neurite extension, and in the presence of an electric field this phenomenon is further encouraged[3]. In the case of PCSA, a non-conducting aerogel substrate with pores on the nanometer scale, the extension of neurites by PC12 cells was further stimulated in the presence of an electric field[3]. In the case of ARF-CA however, we hypothesize that the externally applied potential difference drives a net current (*I*) in the aerogel scaffold as depicted in Figures 4a and 4b. The blue arrows in these Figures indicate the possible current pathways

with an overall direction towards the anode. While an electric field may still exist in the region immediately above the substrate, this is considered to have a negligible effect on neurite extension when compared to the more intense current flowing through the aerogel.

There is no preferential orientation for the neurites extended by PC12 cells plated on electrically conductive amorphous aerogel substrates

In previous studies we showed that PC12 cells grown in the presence of exogenous fields on PCSA showed preferentially extended towards the anode[3]. In this work, however, no preferential orientation was observed. We attribute this lack of directionality to electrochemical polarization of water-saturated superficial pores and the presence of an electrical double layer (EDL) around each pore[29]. The average pore diameter in ARF-CA is ~5 µm (Figure 4a) and it is likely that the pores are filled with culture medium thus creating a two-phase

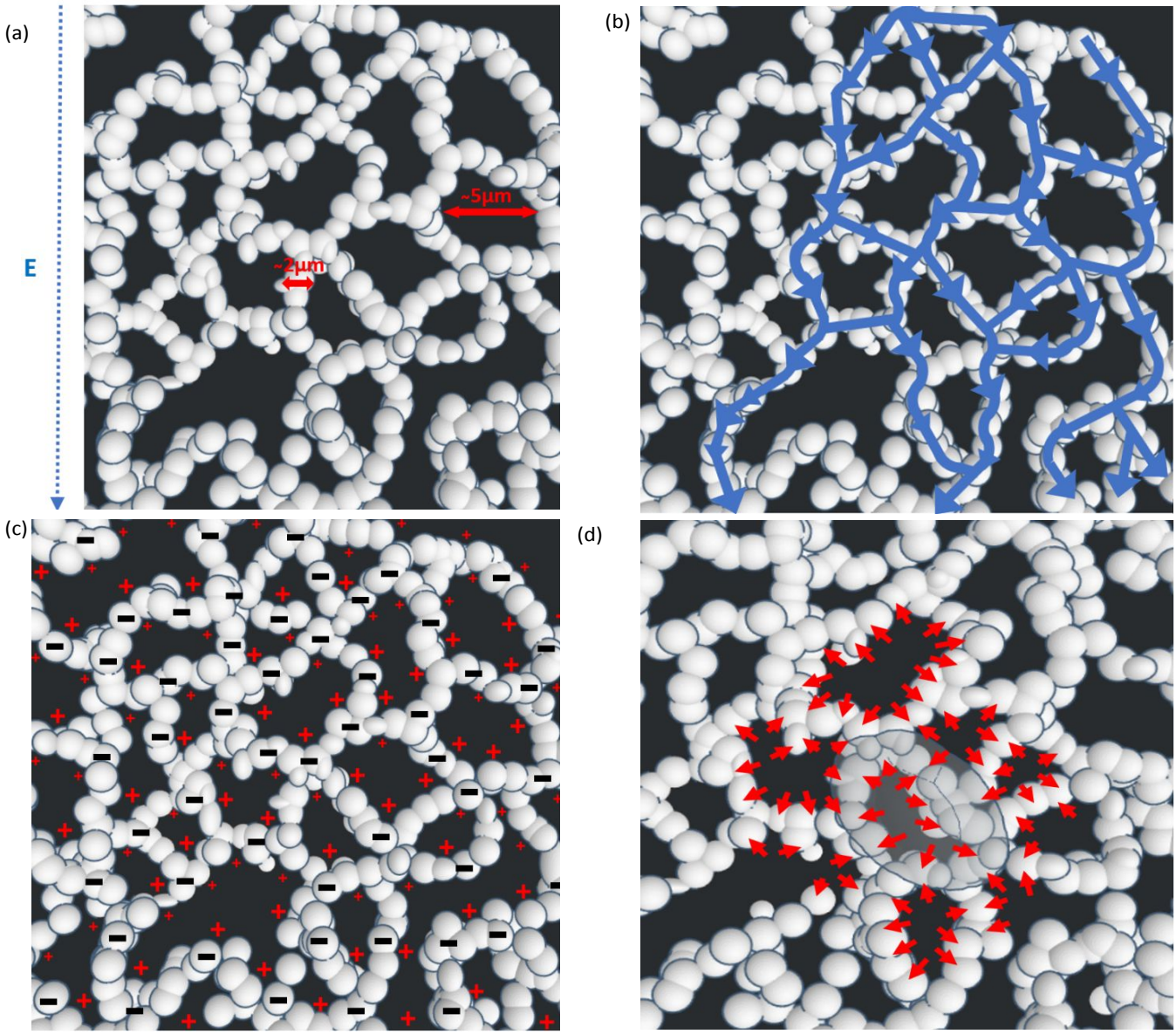

Figure 4: Schematic of the interaction between electric field, aerogel and PC12 cells at the microscopic level. (a) Schematic of the aerogel structure at the microscopic level with the orientation of the external electric field. In white is the ARF-CA structure formed by chains of beads and in black the pores within the structure. (b) Due to the electrically conductive nature of ARF-CA, the structure of this aerogel generates a current (blue arrows) in the presence of an electrical field. (c) Influence of pores and ARF-CA bulk structure on the current induced by an external field. Due to the conductive nature of the ARF-CA and dielectric nature of the pores, a positive charged layer is formed in the pores surrounding the ARF-CA structure. (d) PC-12 cells sense the induced electric current from the outermost layer of the pores and the CA-ARF structure in every direction, which may explain why they extend neurites in all directions.

system with a liquid-solid boundary at the pore level.

We hypothesize that when the electrically conducting aerogel, immersed in cell culture medium, is placed within the stimulation chamber, an induced current I flows through the

ARF-CA structure. Using Ohms law, the current *I* can be approximated as:

$$I = \frac{V}{R} \quad (1)$$

Where resistance is defined as:

$$R = \frac{1}{\sigma}\frac{l}{A} \quad (2)$$

Substituting (2) in (1) provides us with an approximate value for the current *I* determined by the following relationship:

$$I = \frac{V\sigma A}{l} \quad (3)$$

The value for resistivity (σ) of the ARF-CA used in Equation 3 was previously measured and is provided in Table S1 and Table 2. The voltage value (*V*) used in this calculation was obtained from earlier published work[2] where COMSOL Multiphysics was utilized to calculate the actual available potential difference (V_{eff}) for the system used in this study. The cross-sectional area (*A*) in Equation 3 corresponds to the solid area only and not the open void space. The total area occupied by the filamentous network of primary Carbon particles, a_{solid} was used in the final calculation (Figure S2). Therefore, Equation 3 can be rewritten as:

$$I = \frac{V_{eff}\sigma a_{solid}}{l}$$

The carbon filaments were assigned a radius of 1 µm (obtained from SEM images) (Figure 4a). For the 5 mm × 5 mm × 2 mm (w × l × t) substrates used in this study, we calculated that approximately n=3.2x10^6 filamentous chains were present in this volume, which corresponds to a total solid area of $a_{solid} = n \times a_{chain} = n(\pi r^2) = (3\times10^6)\ \pi\ (10^{-6})^2 = 0.09 cm^2$. With these values, a current *I* of 2 A and 4 A was generated at 15 V and 30 V, respectively (Table 2). These values are similar to those shown in section 2.1, showing a current though the ARF-CA sample around 1 A. Due to the heterogeneous nature of the ARF-CA (two phases with different electrical properties; ARF-CA structure and pores), when current is induced by an external electric field, an interfacial polarization occurs at the interface between the pores and the bulk of the aerogel. The mismatch of electrical properties causes the current density at the interface to be different resulting in accumulations of charges at the interface[28]. Figure 4c shows the interaction between the two phases of the aerogel, and the accumulation of charges at the interface. This accumulation of charges induces smaller electric fields with random orientations around the cells, which is likely to be responsible for the random orientation of the neurite extended by PC12 cells. Figure 4c shows the interaction of PC12 cells with the induced electric fields throughout the ARF-CA structure

Table 2: Values used in Equation 3

$a_{solid} = n\ (a_{chain})$	σ	V_{eff}	l	*I*
0.09 cm^2	10 S/cm	1 V	5 mm	2 A
0.09 cm^2	10 S/cm	2 V	5 mm	4 A

Conclusion

The work presented here adds another layer of knowledge about the influence of external bias on neurite extension by neuronal cells. The work focuses on the neurite extension by neuronal cells plated onto an aerogel subjected to an electrical bias (DC). Neurite length increased with increasing voltage in the 0-30 V range and the neurites were much longer in ARF-CA (43% and 106%) compared to TCPS (6% and 15%) and PCSA (12% and 20%) non-conductive substrates.

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Author Contributions

Conceived, supervised and funded the project and wrote the manuscript (with support from O.S., M.W. M.R.S. and S.C.), F.S.; performed the experiments and was responsible for most of the data analysis, M.R.S.; contributed reagents/materials/analysis tools and contributed to interpretation of results, O.S.; contributed reagents/materials/analysis tools, M.W and S.C. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors report no conflicts of interest.

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