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Developing a tissue-engineered neural-electrical relay using encapsulated neuronal constructs on conducting polymer fibers

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Abstract
Neural-electrical interface platforms are being developed to extracellularly monitor neuronal population activity. Polyaniline-based electrically conducting polymer fibers are attractive substrates for sustained functional interfaces with neurons due to their flexibility, tailored geometry and controlled electro-conductive properties. In this study, we addressed the neurobiological considerations of utilizing small diameter (<400 μm) fibers consisting of a blend of electrically conductive polyaniline and polypropylene (PA–PP) as the backbone of encapsulated tissue-engineered neural-electrical relays. We devised new approaches to promote survival, adhesion and neurite outgrowth of primary dorsal root ganglion neurons on PA–PP fibers. We attained a greater than ten-fold increase in the density of viable neurons on fiber surfaces to approximately 700 neurons mm^{-2} by manipulating surrounding surface charges to bias settling neuronal suspensions toward fibers coated with cell-adhesive ligands. This stark increase in neuronal density resulted in robust neuritic extension and network formation directly along the fibers. Additionally, we encapsulated these neuronal networks on PA–PP fibers using agarose to form a protective barrier while potentially facilitating network stability. Following encapsulation, the neuronal networks maintained integrity, high viability (>85%) and intimate adhesion to PA–PP fibers. These efforts accomplished key prerequisites for the establishment of functional electrical interfaces with neuronal populations using small diameter PA–PP fibers—specifically, improved neurocompatibility, high-density neuronal adhesion and neuritic network development directly on fiber surfaces.

(Some figures in this article are in colour only in the electronic version)

Introduction

Neurobiological-electrical interface platforms are being developed and implemented to extracellularly probe and modulate the function of neuronal populations for both *in vitro* and *in vivo* applications. These specialized systems are in widespread use in neural cell cultures, brain slices, and even in the intact brain, and permit continuous monitoring of neuronal activity over weeks or months. Commercially available multi-electrode array (MEA) systems are made for *in vitro* experimentation and consist of rigid electrode-bearing surfaces arranged in a planar or a dimpled-shaped three-dimensional (3D) orientation (Martinoia *et al* 1993, Potter and DeMarse 2001, Morin *et al* 2005, Nam *et al* 2006). For interfacing with the nervous system of living organisms, arrays of sharp penetrating electrodes have traditionally been used to record neuronal population signaling. These systems have classically involved rigid metal and/or silicon electrodes; however, microstrains generated in neural tissue immediately adjacent to implanted electrodes typically exacerbate the inflammatory response and scar formation, potentially contributing to signal attenuation in chronic applications (Lee *et al* 2005, McConnell *et al* 2007). Thus, mechanical compliance is of utmost importance for sustained interface systems, and there has been recent interest in developing more flexible electrodes to mitigate detrimental effects due to the mismatch of mechanical properties (Patrick *et al* 2006).

Electrically conducting polymer (ECP) fibers are attractive alternative substrates for sustained functional interfaces with neuronal populations due to their relative flexibility (compared to metal or silicon), modifiable geometry and chemistry, and controlled electro-conductive properties (Wong *et al* 1994, Richardson-Burns *et al* 2007). One such ECP is polyaniline, which is particularly advantageous because of its ease of fabrication and low cost. The degree of electrical conductivity in polyaniline is determined by deliberate 'doping' with a proton donor to transform the non-conductive emeraldine base into a conductive emeraldine salt by modifying the oxidative state of the material (Panipol 2008a). In the current study, we utilized an ECP composed of a blend of polyaniline and polypropylene (PA–PP). Conductivity of the final blended PA–PP was achieved through percolation, resulting in a sufficient density of polyaniline fibers to form a conductive pathway. Such polymer fiber blends containing polyaniline have the potential to serve as a controlled electrical interface with neuronal populations.

Regarding general biocompatibility, polyaniline has been assessed *in vivo* following subcutaneous implantation in rats, demonstrating minimal cellular infiltration and fibrous matrix formation by four weeks (Mattioli-Belmonte *et al* 2003) or two years (Kamalesh *et al* 2000) post-implantation. Studies evaluating the ability of electroactive polyaniline materials to support cell adhesion, survival and functionality *in vitro* present mixed results. Aplysia neurons grown directly on polyaniline demonstrated reduced viability and irregular morphology compared to control substrates containing additionally poly-L-lysine (PLL) (Oren *et al* 2004). While cardiac myocytes grew well on polyaniline films, an initial decrease in proliferation was observed (Bidez *et al* 2006). On the other hand, a polyaniline derivative modified with cell-adhesion molecules was found to increase cell adhesion, proliferation and neurite outgrowth using the neuronal-like PC-12 cell line (Guo *et al* 2007). Collectively, these studies suggest that polyaniline was not overtly cytotoxic, but did require pro-adhesive surface modifications to support cell adhesion, survival and functionality. Notably, these studies utilized polyaniline-based films on planar substrates (e.g., glass, polystyrene). In contrast, in our application we are utilizing micro-scale 3D conductive polymer fibers (<400 μm in diameter) that contain polyaniline as the electroactive component. Additionally, methodology to culture networks of primary mammalian neurons directly on polyaniline-based micro-fibers has not been established.

Our long-term objective is to develop and implement nervous tissue interface platforms consisting of arrays of mechanically compliant (i.e., flexible) electrodes embedded in 3D neural constructs for use in the central nervous system (e.g., retinal prosthesis) or peripheral nervous system (e.g., robotic prosthetic limb). Such technology may serve as an important component for a biohybrid neural interface microsystem to be used as investigative platforms or as neurobiologically active electrical relays (figure 1). Thus, our goal is to engineer 3D neural cellular constructs (Cullen *et al* 2007a, 2007b), with the added element of electrical functionalization via construct formation around a conductive backbone. The premise behind these living tissue-engineered neural relays is that a stable electrical interface may be formed *in vitro* prior to implementation *in vivo*. Host integration may occur with the biological component of this biohybrid platform, thus exploiting a more natural interface (Pfister *et al* 2007). For example, in the case of limb amputation, it may be desirable to integrate with the peripheral nervous system in order to avoid implanting electrodes into the otherwise non-injured brain while providing the ability to utilize the totality of the exquisite processing power of the brain. However, in this case, peripheral nerve axons will require a *living* target for innervation, hence the use of *living* tissue-engineered relays. Additionally, forming stable interfaces *in vitro* prior to *in vivo* integration may mitigate several of the factors believed to contribute to performance degradation of chronically implanted electrodes (Merrill and Tresco 2005).

In the present study, we developed novel tissue-engineering techniques incorporating ECP strands to create prototype neural constructs for use as living neural-electrical relays. Specifically, we assessed the neurobiological considerations of utilizing PA–PP fibers as the conductive backbone in these novel tissue-engineered relays by addressing (1) cytotoxicity and mitigation strategies, (2) neuronal adhesion, survival, neurite outgrowth and (3) construct encapsulation. The scope of future studies will be to leverage the tailored electro-conductive properties of ECP fibers to extracellularly stimulate neuronal populations intimately adhered to the material surface following hydrogel encapsulation.

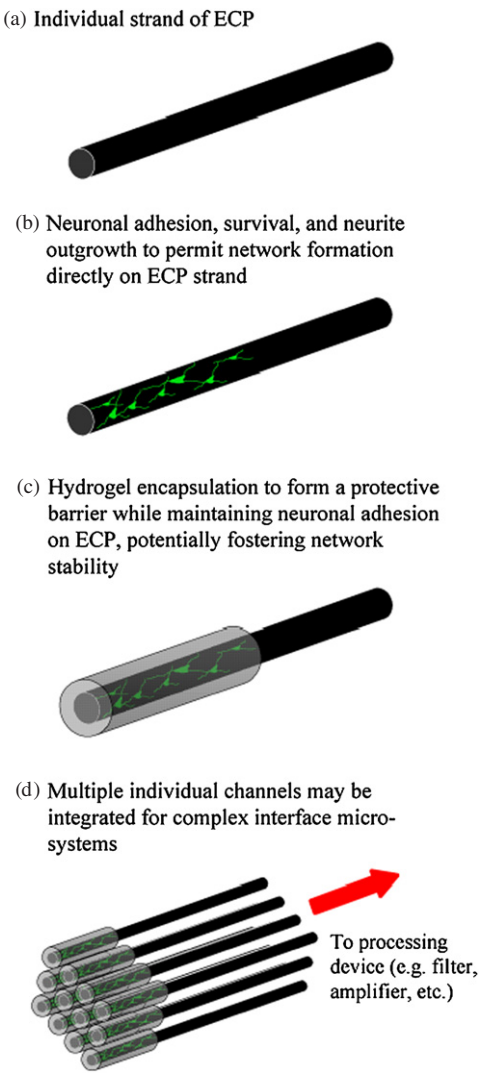

Figure 1. Tissue-engineered neural-electrical relays. Small diameter (<400 μm) electrically conducting PA–PP fibers were chosen as the electrical interface due to their mechanical compliance and tailored conductive properties (a). PA–PP fibers were processed to permit neuronal growth and network formation directly on the fibers (b). Then, these biohybridized neural-conductive fibers were encapsulated with a bio-inert hydrogel to form a protective barrier while potentially stabilizing the network in intimate contact with the PA–PP (c). A multitude of relays may be used in parallel to create complex interface microsystems, with the alternate end of the PA–PP fiber projecting to a processing device for signal filtering, amplification and output (d). These 'wet' biohybrid neural interface systems may be used as investigative platforms *in vitro* or as biohybrid relays *in vivo*.

Materials and methods

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996). All components were from Invitrogen (Carlsbad, CA) or BD Biosciences (San Jose, CA) unless noted.

Electrically conductive polymer

The ECP material used in the current study was generated by Solutia, Inc. using a proprietary process. ECP material was a melt-processed blend of electrically conductive polyaniline (Panipol® CX, Panipol Oy, Finland) (Panipol 2008b) and polypropylene (PA–PP) mixed in a controlled ratio. Fibers were manufactured by extrusion through a capillary rheometer at varying shear rates. Conductivity of the final blended PA–PP was achieved through a sufficient percolation density of polyaniline to form a conductive pathway and varied with shear rate (Panipol 2008b). Following manufacturing, the fiber was reported to have a resistivity of at least 5.2 Ω cm (communication from Solutia, Inc.).

Neuronal cell culture

Dorsal root ganglia (DRG) were isolated from E15 Sprague-Dawley rats (Charles River, Wilmington, MA). DRG explants were isolated in L-15 medium and dissociated using trypsin (0.25%) + EDTA (1 mM) for 1 h at 37 °C. Neurobasal medium + 5% FBS was then added, and the tissue was triturated followed by centrifugation at 1000 rpm for 5 min. The supernatant was aspirated and the cells were resuspended at 5×10^6 cells ml^{-1} in neurobasal medium supplemented with 2% B-27, 500 μM L-glutamine, 1% Penicillin/Streptomycin, 1% FBS, 2 mg ml^{-1} glucose (Sigma, St. Louis, MO), 10 ng ml^{-1} 2.5S nerve growth factor, 10 μM FdU (Sigma) and 10 μM uridine (Sigma). DRG were plated by placing 100 μL of cell suspension either adjacent to the PA–PP fibers/sheets or directly on the PA–PP fibers. The cultures were placed in a humidified tissue culture incubator (37 °C and 5% CO_2) for 3 h to allow cells to attach, after which, media were added to the culture vessel. The culture media were changed every 2–3 days *in vitro* (DIV) by replacement with fresh pre-warmed media.

PA–PP preparation and surface treatments

PA–PP sheets (1 cm × 1 cm × 170 μm) or fibers (1 cm length × 390 μm diameter) were either left undisturbed or soaked in de-ionized (DI) water or culture media (at 37 °C) for one week prior to culture. Prior to cell plating, PA–PP samples were rinsed in DI water for 15 min on a shaker, sterilized by soaking in 70% ethanol for 15 min on a shaker, and rinsed again with sterile DI water for 15 min. The samples were then air dried in a laminar flow hood.

Surface modifications are necessary to facilitate the adhesion and growth of DRG neurons on glass and polystyrene as well as on PA–PP fibers. In this study, surface modifications included either poly-L-lysine (PLL) (0.02 mg ml^{-1}, Sigma) alone or PLL plus collagen type I (3.5 mg ml^{-1}, Becton Dickinson). The samples were treated with PLL overnight, rinsed with sterile cell culture water and air dried in a laminar flow hood. When collagen was added, polymerization occurred by exposure to ammonia vapors for 3 min followed by air-drying for 1–2 h. When PA–PP fibers were coated in the culture chambers, collagen was added to a region (∼1 cm^2) and spread over the surface in a thin coat prior to

polymerization/drying (this coated the fiber as well as the local chamber base). Alternatively, PA–PP fibers were selectively coated by dipping in collagen solution (3.5 mg ml^{-1}) followed by polymerizing/drying while suspended.

Neurocompatibility of PA–PP

To evaluate toxicity, neuronal cultures were plated in polystyrene cell culture dishes immediately adjacent to the PA–PP sheets, the PA–PP fibers, or in control wells without PA–PP material. Prior to plating, the PA–PP sheets or the fibers were attached to tissue culture dishes with medical grade RTV silicone adhesive (NuSil Silicone Technology, Carpinteria, CA). A region ($\sim$1 cm^2) immediately adjacent to the adhered PA–PP sheet/fiber (or in the center of the well in the case of controls without PA–PP) was treated with PLL followed by collagen (50 μl). Neuronal survival was evaluated following plating adjacent to (1) non-pre-soaked PA–PP, (2) pre-soaked PA–PP, or (3) absent PA–PP but fed media previously used to soak PA–PP. Viability was assessed using a fluorescent probes to distinguish live and dead cells (LIVE/DEAD viability/cytotoxicity kit; molecular probes, Eugene, OR). Briefly, cell cultures were rinsed in phosphate buffered saline (PBS) and incubated with 2 mM calcein AM and 4 mM ethidium homodimer-1 at 37 °C for 30 min and then rinsed in PBS. Initial neurocompatibility studies used non-pre-soaked PA–PP, with neuronal cultures plated adjacent to the PA–PP sheets, the PA–PP fibers, or in control wells without PA–PP material and culture viability was assessed at 28 DIV ($n = 3$ cultures per group). For detoxification studies, neuronal cultures were plated adjacent to the unaltered PA–PP sheets ($n = 7$), the pre-soaked PA–PP sheets ($n = 7$), or absent PA–PP but fed media in which the PA–PP sheets had previously been soaked ($n = 3$) and culture viability was assessed at 7 DIV. To assess viability, the numbers of live and dead cells were manually quantified across sampled regions.

Neuronal adhesion and growth on PA–PP fibers

Our goal was to develop methodology to facilitate the formation of integrated neuronal networks directly adhered to PA–PP fibers. To accomplish this, neuronal cultures were plated in custom culture chambers consisting of glass slides with polydimethylsiloxane containment rings (PDMS; Sylgard 184, Dow Corning, Midland, MI). PA–PP fibers were in one of the two configurations: either (1) adhered to the culture well base with an RTV silicone adhesive, referred to as 'adhered' fibers; or (2) suspended approximately 200–300 μm above the chamber base by being fed through a small orifice on the side of the PDMS containment rings, which was then sealed with the RTV silicone adhesive to prevent leaking, referred to as 'suspended' fibers. 'Suspended' fibers were above either PLL treated or non-PLL treated glass chambers bases. To evaluate neural cell adhesion, growth and distribution directly on PA–PP fibers, cell suspensions from dissociated DRG were plated directly above pre-soaked PA–PP fibers (or in the center of the chamber ins the case of controls without PA–PP).

At 7 DIV, the density and distribution of neuronal somata adhered to the PA–PP fibers were assessed via immunocytochemistry (ICC). Briefly, the cultures were fixed in 4.0% formaldehyde for 30 min, rinsed in PBS and permeabilized using 0.1% Triton X100 plus 4% goat serum for 20 min. Primary antibodies were added (in PBS + 4% serum) at 37 °C for 4 h. Primary antibodies for the following neuronal cytoskeletal proteins were used: (1) MAP-2 (SMI-52R, 1:1000, Covance, Princeton, NJ), a microtubule-associated protein expressed primarily in neuronal somata and dendrites, and (2) tau (A0024, 1:400, Dako, Carpinteria, CA), a microtubule-associated protein expressed primarily in axons. After rinsing, secondary fluorophore-conjugated antibodies (Alexa 488- or 546-conjugated IgG; molecular probes) in PBS plus 4% serum were added at 18–24 °C for 2 h. Neuronal adhesion and distribution were initially assessed on detoxified 'adhered' PA–PP fibers following PLL/collagen surface treatment ($n = 7$) in comparison to control cultures absent PA–PP ($n = 3$). Neuronal adhesion was then assessed on detoxified 'suspended' PA–PP fibers following PLL/collagen surface treatment either above PLL treated ($n = 5$) or non-PLL treated ($n = 5$) culture chambers. The density of neuronal somata (MAP-2^+) adhered to PA–PP fibers was manually quantified over sampled regions along fibers.

Agarose encapsulation

Neuronal cultures were encapsulated in SeaPrep agarose (BioWhittaker Molecular Applications, Rockland, ME) at 6–9 DIV. The agarose was prepared at 4% (w/v) in PBS and then mixed with media in appropriate concentrations to generate gels at 0.5%, 1.0%, 1.5% and 2.0%. These gel solutions were maintained at 37 °C prior to the introduction of the PA–PP fiber/culture. Encapsulation occurred by containing the PA–PP fibers using custom cylindrical chambers generated from polycarbonate and/or PDMS and slowly adding the warmed (semi-fluid-like) agarose around the PA–PP fibers. The entire apparatus was transferred to −20 °C for 60 s for rapid cooling and then 4 °C for 20 min to assure gelation. For encapsulation studies, culture viability was assessed (as previously described) approximately 60 min following gelation at agarose concentrations of 0.5–1.0% ($n = 3$) or 1.5–2.0% ($n = 5$) compared to non-encapsulated controls ($n = 3$).

Phase contrast and confocal microscopy

Cell cultures and PA–PP constructs were imaged using phase contrast and confocal microscopy techniques, each using Eclipse TE300 microscopes (Nikon, Melville, NY). Phase contrast images were digitally captured and analyzed (Spot RT Color; Diagnostic Instruments, Sterling Heights, MI). Following immunocytochemistry and viability/cytotoxicity staining, cells were fluorescently imaged using a laser scanning confocal microscope (BioRad Radiance 2000-MP; Zeiss, Oberkochen, Germany). Multiple z-stacks (2.5–10 μm plane-to-plane separation) were acquired across the full thickness of cultures from the various culture conditions and time points. Z-stacks that included PA–PP fibers were $\geqslant$200 μm thick.

Statistical analyses

General linear model ANOVA was used with PA–PP condition, surface treatment, or encapsulation scheme as independent variables and culture viability or neuronal density as dependent variables. When significant differences were found between groups, post hoc Tukey's pair-wise comparisons were performed. For all statistical tests, $p < 0.05$ was required for significance.

Results

Neuronal survival and growth adjacent to PA–PP

We assessed the neurocompatibility of blended polyaniline–polypropylene (PA–PP) by evaluating neuronal survival following plating adjacent to the unmodified PA–PP sheets or fibers secured to the base of a culture dish. Planar PA–PP sheets were used in addition to fibers in order to increase the ratio of material to media, thus assessing neurocompatibility under potentially more strenuous conditions. Standard polystyrene tissue culture wells without the presence of PA–PP served as controls.

At 2 DIV, neuronal cultures in control wells absent PA–PP appeared healthy, and by 12 DIV, exhibited robust neurite outgrowth (figure 2). However, in cultures adjacent to the PA–PP sheets, there was widespread cell death by 2 DIV, signifying that neuronal death occurred rapidly in this case. Indeed, by 12 DIV, there was almost complete neuronal death in cultures containing PA–PP sheets. In the case of PA–PP fibers, cell death in immediate proximity of the fibers was observed at both 2 DIV and 12 DIV, resulting in a substantial decrease in the density of neuronal somata immediately adjacent to the PA–PP fibers (figure 2). At 28 DIV, control cultures remained viable and no further cell death was observed in the proximity of the fibers. Thus, toxicity was limited to within the first few days of culture, potentially diluted by subsequent media changes. Additionally, a relatively wide (200–800 μm) gap adjacent to the PA–PP fibers was maintained with a near complete absence of viable neuronal somata. Interestingly, apparently after the toxic effects ceased, robust neurite outgrowth progressed toward the PA–PP fibers, which appeared to be preferentially oriented orthogonal to the fibers, and then progressed longitudinally along the fibers (figure 2).

While total cell death occurred immediately adjacent to the PA–PP sheets, cell viability improved with increasing distance from the sample. For instance, there was consistently complete cell death immediately adjacent to the PA–PP sheets, but viability improved to over 60% by 3.0 mm away from the sheets (figure 3). Thus, the detrimental effects on neuronal viability were dependent upon the quantity of PA–PP per unit volume and the neuronal distance from the PA–PP, suggesting the leaching and subsequent diffusion of an agent detrimental to neuronal survival.

To test this hypothesis, we attempted to detoxify PA–PP by soaking in media and then culturing neurons adjacent to these pre-soaked PA–PP. Detoxifying PA–PP sheets resulted in DRG neuronal viability of 89.2 $\pm$ 11.3%, which was a significant improvement over a neuronal viability of <1% found adjacent to the non-soaked PA–PP ($p < 0.001$) (figure 3). To further demonstrate the alteration of culture media by a toxic leachant, neuronal cultures (absent PA–PP) were fed with media previously used to soak the PA–PP sheets. Here, survival was <5%, which did not statistically vary from the viability adjacent to the untreated PA–PP sheets. Given that the pre-soak media itself was toxic to neurons, this further substantiated the presence of a toxic agent emanating from the PA–PP.

Formation of integrated neuronal networks on collagen-coated PA–PP fibers

Our primary objective was to promote the *preferential* adhesion and growth of neuronal somata and neurites directly along the PA–PP fibers to facilitate the formation of integrated neuronal networks in intimate contact with the ECP. This is important since intimate contact will be required in future applications to produce an electrical coupling between the PA–PP and the neuronal network. First, we assessed the ability of functionalized surface modifications to improve neuronal adhesion and distribution on detoxified PA–PP fibers. Specifically, at 7 DIV, neuronal survival and growth were assessed via ICC for two neuronal-specific cytoskeletal proteins, the somatic protein MAP-2 and the axonal protein tau. Control cultures were plated on PLL/collagen-coated glass absent PA–PP, revealing a fairly homogeneous distribution of small clusters of neuronal somata on the glass surface (figure 4). As a starting point, detoxified PA–PP fibers were adhered to glass and treated with PLL/collagen in place, thus treating both the fiber and the underlying substrate. In this case, the neuronal distribution was non-homogeneous with few cell bodies adhered to the fibers (<100 neurons mm^{-2}). Surprisingly, even when a collagen coating was selectively applied to PA–PP fibers by dipping in collagen solution, the neuronal density on the fibers remained exceedingly low (figure 4). Thus, proteinaceous surface treatment (adsorption) alone did not improve neuronal adhesion, and hence density, on the fibers. Interestingly, projected tau$^+$ axons appeared to adhere preferentially to the fibers, as noted previously, underscoring differences in optimized parameters for neuronal adhesion versus neurite outgrowth. This preferential axonal growth may be related to mechanical (i.e., increased stiffness) or geometric (i.e., curvature) parameters (Smeal *et al* 2005, Yu and Shoichet 2005, Bellamkonda 2006).

We were able to significantly improve the density of neuronal somata adhering directly to the PA–PP fibers by both attracting cells to the fiber and at the same time repelling the cells from the base substrate. Specifically, the fibers were suspended ~200–300 μm above hydrophobic (non PLL-treated) glass rather than hydrophilic (PLL-treated) glass. When suspended above hydrophilic glass, the cell density of neurons (as assessed by MAP-2/tau ICC) on PA–PP fibers was 60.8 $\pm$ 9.3 neurons mm^{-2}. By suspending the PA–PP fibers above hydrophobic glass, the neuronal density was increased by a statistically significant margin to 691.8 $\pm$ 457.7 neurons mm^{-2} ($p < 0.05$). The high

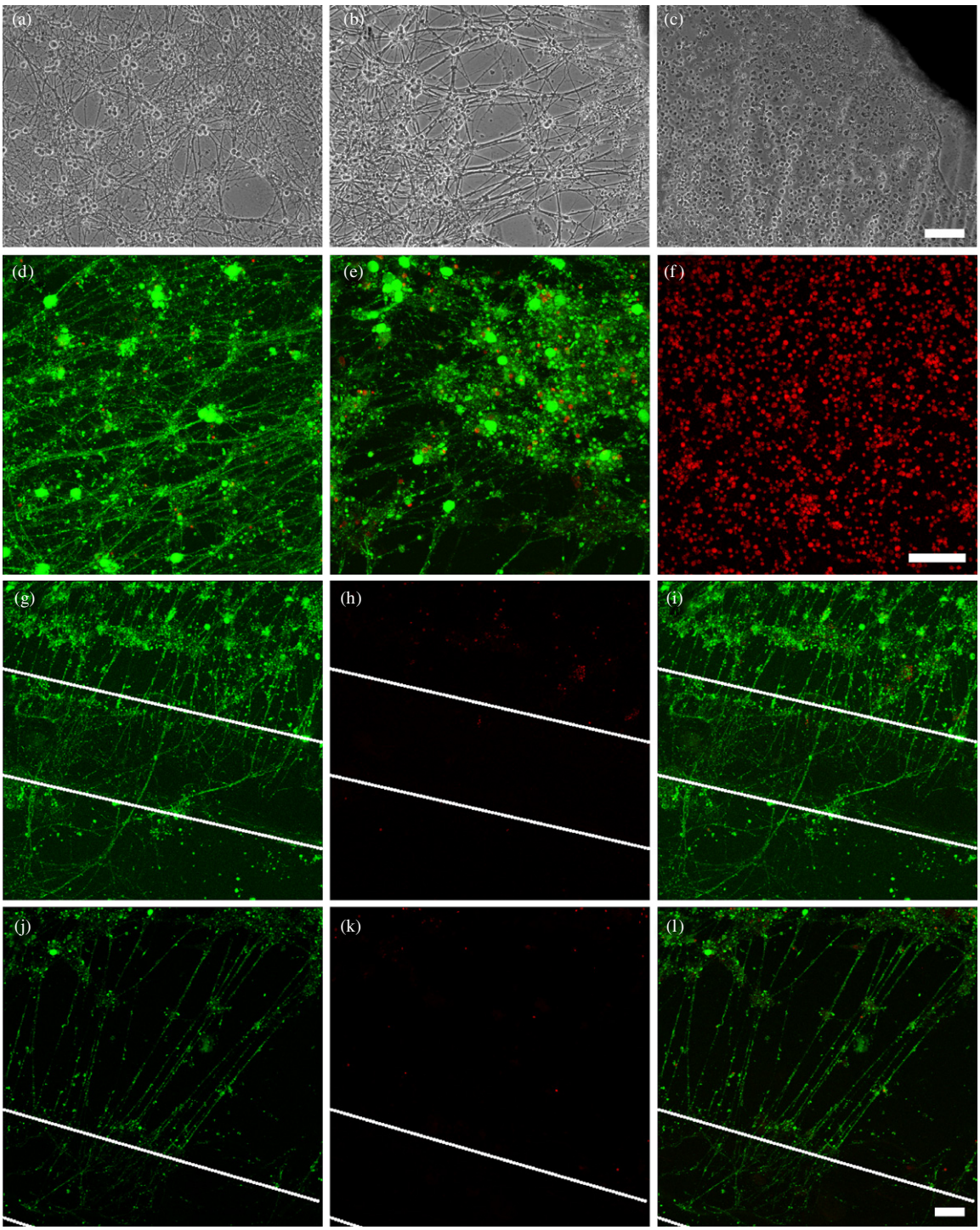

Figure 2. Neuronal survival and growth adjacent to PA–PP. Representative phase contrast photomicrographs of neuronal cultures plated on polystyrene without PA–PP (a), adjacent to the PA–PP fiber (b) and adjacent to the PA–PP sheets (c) at 12 DIV. Representative confocal fluorescent micrographs of neuronal cultures labeled to discriminate live cells (green) from dead cells (nuclei labeled red) plated either on polystyrene without PA–PP (d), adjacent to the PA–PP fiber (e); fiber located at the bottom of the image, just outside field of view, and adjacent to the PA–PP sheets (f) at 28 DIV. Neuronal cultures exhibited high viability and neurite outgrowth absent PA–PP (a), (d). Few neuronal somata were observed directly adjacent to the PA–PP fibers (b), (e). Widespread neuronal death was observed in cultures containing PA–PP sheets (c), (f). Confocal fluorescent micrographs from neuronal cultures plated adjacent to the PA–PP fibers showing live cells (green); (g), (j) and dead cells (nuclei labeled red); (h), (k) at 28 DIV; with overlay (i), (l) (PA–PP edges denoted by white lines). There was a gap of 200–800 μm from the PA–PP with a near complete absence of viable neuronal somata; however, neurite outgrowth progressed orthogonal toward the PA–PP fibers and then progressed along the fibers. The scale bars = 100 μm.

variability in cell density on the fibers was due to longitudinal sampling, where there were alternating regions of high and relatively low neuronal somata density spanned by regions of continuous neuritic tracts. Indeed, neuronal somata-rich

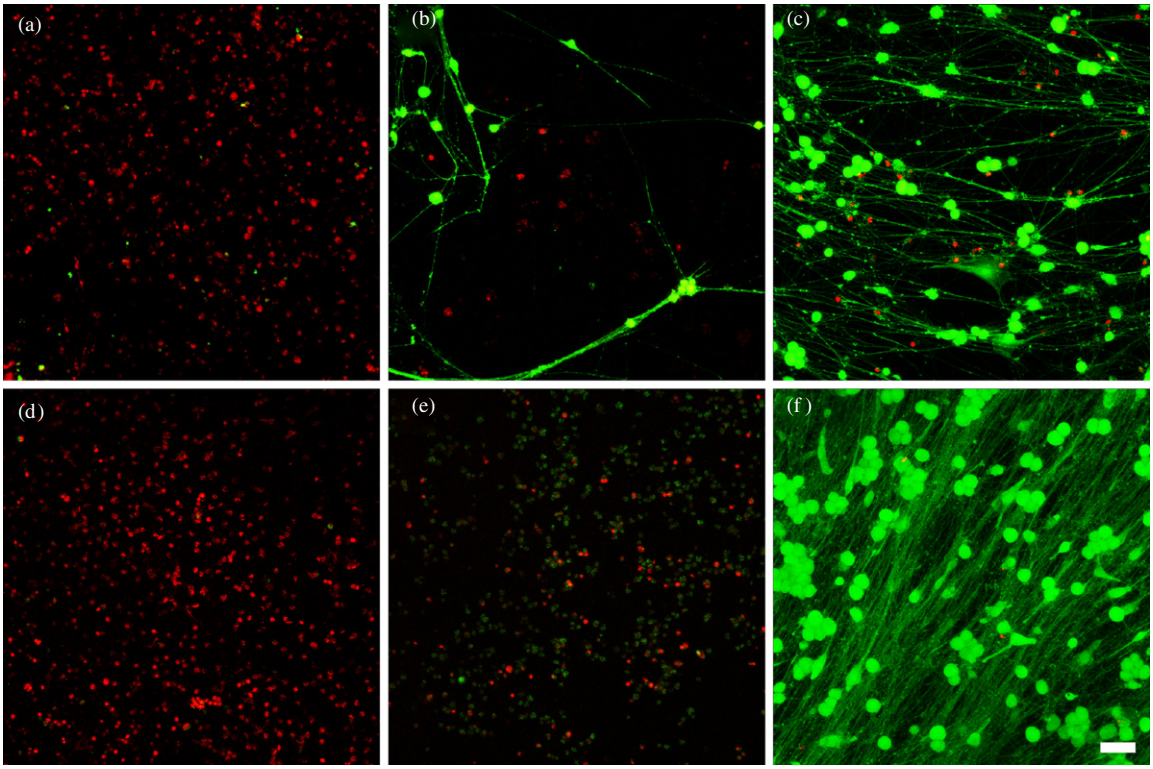

Figure 3. Neuronal survival adjacent to the PA–PP sheets. Representative confocal fluorescent micrographs of neuronal cultures at 7 DIV (live cells labeled green and nuclei of dead cells labeled red). In neuronal cultures plated adjacent to untreated PA–PP, viability was extremely poor directly adjacent (a), but improved at 1.5 mm away (b) and improved further at 3.0 mm away (c). Thus, neuronal survival improved with increasing distance from the PA–PP, suggesting leaching and diffusion of a substance from the PA–PP detrimental to neuronal survival. To test this hypothesis, neurons were plated adjacent to untreated PA–PP (d), plated absent PA–PP but fed media that PA–PP was soaked in previously (e), or plated adjacent to PA–PP previously soaked in media (f). Neuronal survival in culture with untreated PA–PP or fed media PA–PP was soaked in was very poor. However, PA–PP was rendered neurocompatible by pre-soaking. The scale bar = 50 μm.

regions had cell densities greater than 1300 neurons mm^{-2}. Possibly, the placement of hydrophobic rather than hydrophilic glass improved initial neuronal adhesion by biasing how the suspension of dissociated cells settled during the initial plating. In turn, this served to improve survival and longer-term growth of neuronal somata and neurites directly on the fibers. Overall, this more than a ten-fold increase in neuronal adhesion directly to PA–PP fibers would provide a suitable neuronal density for functional integration with the neuronal population.

Hydrogel encapsulation of neuronal networks on the PA–PP fibers

The encapsulation of integrated neuronal networks adhered to the PA–PP fibers *in vitro* may stabilize the network while forming a protective barrier and support structure useful upon subsequent implantation *in vivo*. Here we considered the effects of encapsulating the fibers and integrated neuronal cultures using a thermo-regulated agarose hydrogel of varying stiffness (proportional to % agarose) (Balgude *et al* 2001, Cullen *et al* 2007a). Neurons were plated on detoxified, collagen-coated, suspended PA–PP fibers and, following network development over 6–9 DIV, the fibers and the adhered neuronal cultures were encapsulated. Under optimized agarose concentrations, encapsulation did not significantly reduce network viability. Specifically, cell viability following 0.5–1.0% agarose encapsulation was 86.1 $\pm$ 6.3%, which did not statistically vary from the viability of 93.6 $\pm$ 1.5% found in non-encapsulated controls (figure 6). However, agarose encapsulation at 1.5–2.0% was not successful, as the entire neuronal networks were consistently sheared off of the fiber surfaces (data not shown). Potentially, these higher concentration hydrogels surpassed a stiffness threshold, creating a mechanical mismatch rendering these hydrogels non-compliant with any micro-motions occurring while transferring the PA–PP fibers. Nonetheless, long PA–PP fibers with neuronal networks encapsulated in 0.5–1.0% agarose could be prepared which maintained somata- and neurite-rich segments (figure 6).

Discussion and conclusions

The goal of the current study was to develop enabling technologies for stable tissue-engineered neural-electrical interface microsystems using a novel approach. Specifically, we assessed the neurobiological feasibility of using small diameter (<400 μm) PA–PP fibers as the conductive backbone for living neural-electrical relays. We developed methodology to render the PA–PP fibers suitable for neuronal cell culture, including appropriate pre-processing and bioactive surface modifications. This resulted in optimized neuronal adhesion and neurite outgrowth, enabling network formation directly

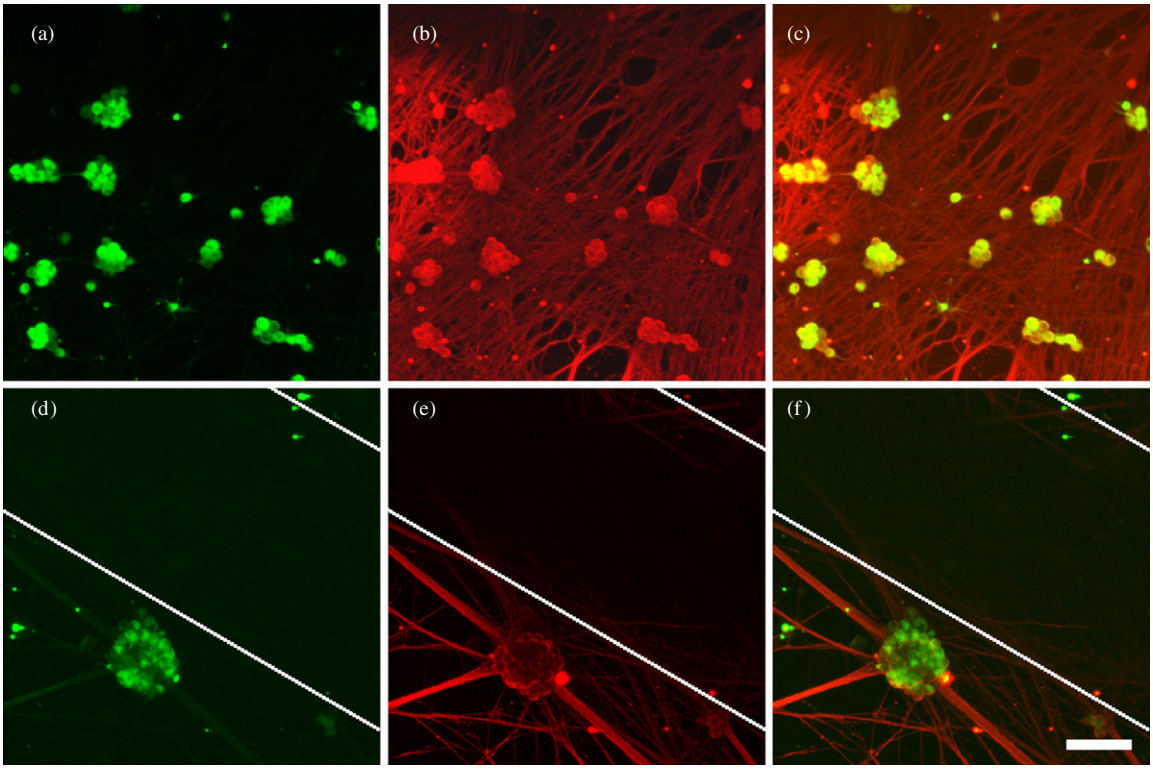

Figure 4. Baseline neuronal adhesion on collagen-coated PA–PP fibers. Representative confocal reconstructions of neuronal cultures at 7 DIV immunolabeled using antibodies recognizing MAP-2 (a), (d), tau (b), (e), with overlay (c), (f). Cultures were plated on pre-treated glass absent PA–PP (a)–(c) or on pre-treated PA–PP fibers above glass (d)–(f) (fiber edges denoted by white lines). When plated without fibers, neurons formed small homogeneously distributed clusters on the glass surface (a)–(c). However, there was a paucity of cell bodies adhered to the collagen-coated fibers, although there was axonal outgrowth across the fibers (d)–(f). The scale bar = 100 μm.

on the micro-scale fibers. This was a key accomplishment for future electrical interfaces, since intimate neuronal integration (adhesion and growth) along the fibers will be necessary to facilitate contiguous extracellular electrical communication with the neuronal population. Finally, these biohybridized neural-conductive fibers were encapsulated within a bio-inert hydrogel to form a protective barrier while potentially stabilizing the network in intimate contact with the PA–PP.

Polyaniline is the electrically conductive component of the ECP used in this study, the conductive properties of which have been known for many years. Polyaniline is initially non-conductive, but may be transformed to the conductive state by increasing the degree of protonation through treatment with an acidic dopant to convert imine groups of the polymer to iminium (Panipol 2008a). Thus, doping of the polyaniline emeraldine base results in a conductive emeraldine salt (Panipol 2008a, Paul *et al* 1985, Hatchett *et al* 1999). Polypropylene, the other component of the blended material in this study, is not conductive.

The cytocompatibility of polyaniline and polypropylene is paramount in determining the suitability for their subsequent use *in vivo*. Previous reports suggest that polypropylene is not excessively inflammatory, and compatible with neuronal survival and neurite outgrowth. For instance, DRG explants were shown to produce robust outgrowth on polypropylene filament bundles coated with fibronectin (Biran *et al* 2001). Additionally, in a peripheral nerve repair model, sciatic nerve regeneration occurred through a polypropylene mesh, although a connective tissue response was observed (Gibson *et al* 1991). Thus, the polypropylene component of the blended material was not expected to contribute to a cytotoxic response *in vitro* or a strong inflammatory response *in vivo*.

Additionally, previous studies have addressed the ramifications of polyaniline implantation *in vivo*. Polyaniline discs were subcutaneously implanted into rats and, at two years post-implantation, the tissue surrounding the implanted polyaniline discs revealed only minor cellular infiltration and collagen fiber formation (Kamalesh *et al* 2000). In a similar study, polyaniline films were implanted subcutaneously into rats and the surrounding tissue was evaluated four weeks later, revealing the formation of a fibrous capsule around the implant; however, the number of inflammatory cells was smallest for polyaniline over other electrically conductive polymers tested (Mattioli-Belmonte *et al* 2003). In both these studies, the tissue responses were minimal and considered acceptable, demonstrating that polyaniline could be chronically implanted into animals without producing major inflammatory reactions or tumor formation.

To gain additional insight into cellular interaction with polyaniline, several *in vitro* studies have evaluated the ability of polyaniline to support cell viability, growth and functionality. Aplysia neurons were grown on planar polyaniline substrates, revealing that neurons grown directly on polyaniline demonstrated a marked reduction in viability

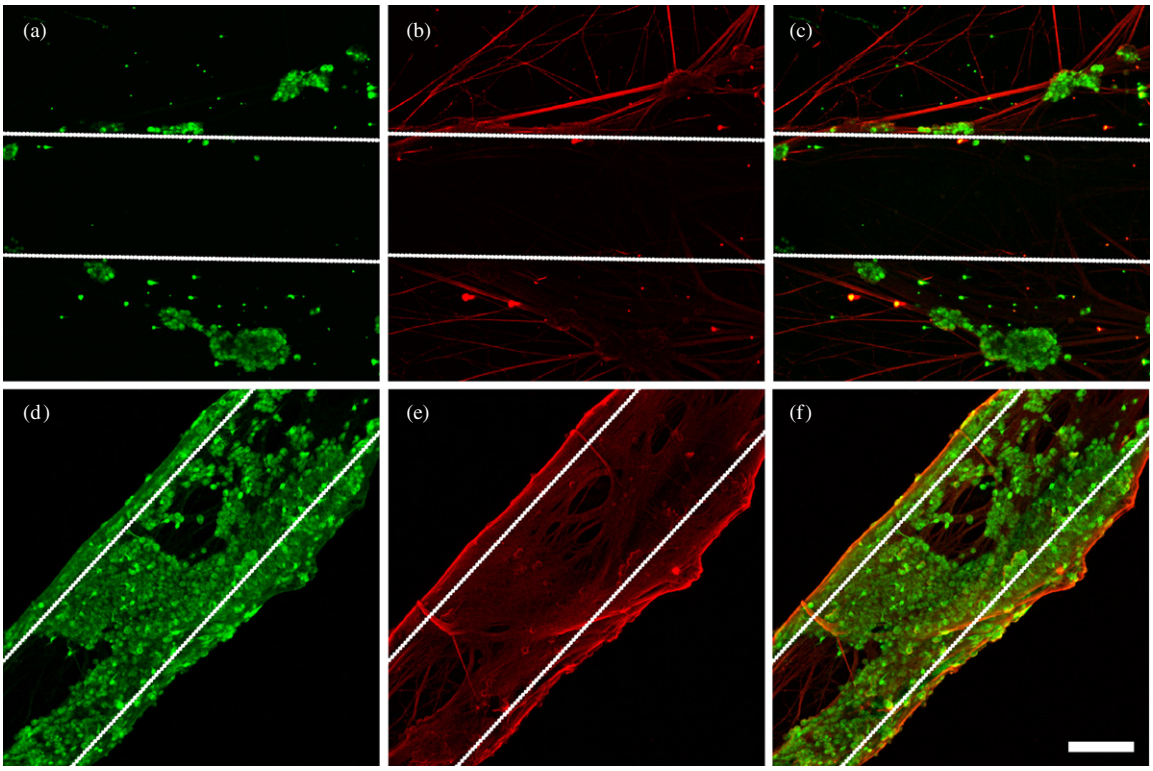

Figure 5. Improved neuronal adhesion on collagen-coated PA–PP fibers. Representative confocal reconstructions of neuronal cultures plated on PA–PP fibers immunolabeled at 7 DIV for MAP-2 (a), (d), tau (b), (e), with overlay (c), (f) (fiber locations denoted by white lines). When fibers were placed above hydrophilic glass, few cell bodies adhered to the fibers (a)–(c); however, when the stands were placed above hydrophobic glass, the neuronal cell density on the fibers increased over 10-fold (d)–(f). The hydrophobic glass surface may have influenced the neuronal descent as the cells settled out of solution upon plating, creating an electrostatic bias toward the hydrophilic collagen-coated fiber. The scale bar = 200 μm.

and irregular morphology compared to control cultures grown with PLL (Oren *et al* 2004). Thus, polyaniline required PLL surface modification to entice neuronal attachment and neurite growth. This was underscored by a subsequent strip assay alternating regions of polyaniline and PLL, revealing that neurons preferentially attached and grew on the PLL strips, avoiding the polyaniline areas (Oren *et al* 2004). Another study considered the growth of cardiac myoblast cells on electrospun fibers of a polyaniline/gelatin blend, demonstrating that the presence of polyaniline in the blend reduced cell attachment (Li *et al* 2006). A different study involving cardiac myocytes evaluated conducting polyaniline (emeraldine salt, E-PANi) versus the non-conducting form (emeraldine base, PANi), revealing decreased proliferation on E-PANi compared to PANi over the first 100 h in culture (Bidez *et al* 2006). Finally, surface modification of polyaniline-derived materials through addition of functional cell-adhesion molecules, which are typically beneficial to foster attachment, survival and outgrowth for neurons in particular, have been investigated (Guo *et al* 2007). In this study, a novel polyaniline-derived material was developed that was functionalized with the oligo-peptide RGD, a prevalent ligand for receptor-mediated cell adhesion. Using the neuronal-like PC-12 cell line, it was demonstrated that surface-bound RGD increased cell adhesion, proliferation and neurite outgrowth.

A conclusion reached in some of these studies was that the de-doping process may be detrimental to cellular function and/or survival (Kamalesh *et al* 2000, Bidez *et al* 2006). De-doping of polyaniline is the result of a deprotonation of the emeraldine salt, releasing protons at the material surface. This release may lower the local pH and thus influence biochemical activity. Although these changes may only occur on the surface of the material, it may cause the polyaniline to lose conductivity. This is particularly relevant in our application, since neuronal adhesion and growth occurs on the surface of the PA–PP fibers. However, the ECP used in the current study was a blend of polyaniline and polypropylene, and the consequences of the potential de-doping process on electrical functionality are not currently known.

Nonetheless, the conclusions of these previous studies are consistent with our findings, where functional polyaniline appeared to release an agent toxic to neurons with detrimental ramifications for survival. Specifically, we found that PA–PP sheets/fibers induced local neuronal cell death over the first two days in culture. Low quantities of PA–PP (fibers) were tolerated by neurons based on distance from the material, potentially as a function of diffusion-related concentrations (Fick's 2nd Law). High quantities of PA–PP (sheets) resulted in rapid and more widespread neuronal death. Thus, PA–PP was not neurocompatible in a quantity- and distance-dependent manner. It is most likely that a chemical reaction on the surface of the PA–PP donated protons to various

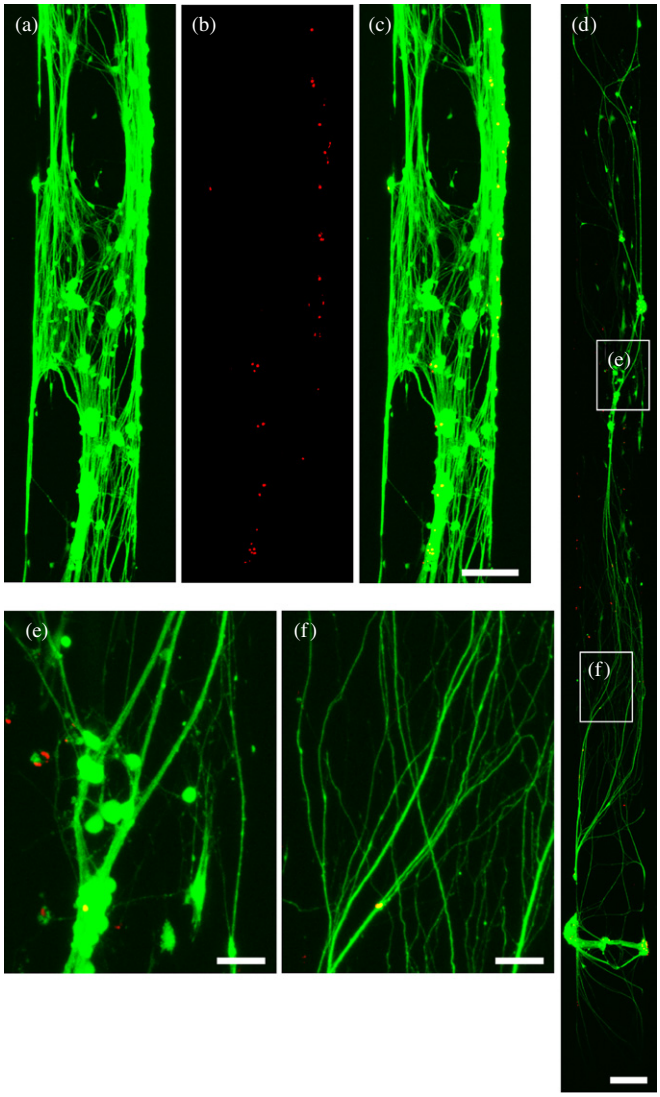

Figure 6. Neuronal encapsulation on PA–PP fibers. Neurons were plated on collagen-coated PA–PP and, at 6–9 DIV, encapsulated using 0.5–1.0% agarose. Representative fluorescent confocal reconstructions of encapsulated neuronal cultures on PA–PP stands stained to discriminate live cells (green) (a) from the nuclei of dead cells (red) (b); with overlay (c) (scale bar = 200 μm). The encapsulation process using 0.5–1.0% agarose did not reduce the cell density or the cell viability versus non-encapsulated controls. An approximately 6.5 mm segment showing encapsulated neuronal network on a PA–PP fiber (d) (the scale bar = 200 μm). Increased magnification of regions of interest from (d) showing a cluster of neuronal somata (e) and a neurite-rich segment (f) following encapsulation (the scale bars = 50 μm).

constituents of the media, making the local microenvironment acidic beyond thresholds for neuronal survival. This, in turn, affected a region proportional to the quantity of material based on the rate of diffusion and the contrary action of the bicarbonate buffering system employed in cell culture. Indeed, the detrimental effects on survival were amenable to detoxification via pre-soaking the PA–PP prior to culture. However, if functional thresholds are surpassed, this de-doping process will result in the polyaniline emeraldine salt to lose conductivity. This creates a situation of competing divergent interests between neuronal survival and PA–PP functionality.

In addition to a favorable biochemical environment, neuronal survival equally depends on the ability to adhere and grow on a biomaterial surface. This is underscored in our application, which required intimate neuronal adhesion, neurite growth and network formation directly on the 3D surfaces of the PA–PP fibers. We were able to achieve this through a combination of pro-adhesive surface treatments and manipulated surface charge differentials to attract settling neuronal suspensions to the surface for subsequent binding. Specifically, we coated the fibers with extracellular matrix proteins (cell-adhesion factors). Then, by carefully suspending the fibers above a hydrophobic surface, we were able to selectively attract cells to the fiber surface as the dissociated neurons settled upon initial plating. Collectively, these efforts developed treatment strategies to improve PA–PP neurocompatibility and facilitate neuronal adhesion and network formation directly on the fibers; however, the ramification of these pre-treatments on PA–PP electro-conductivity will need to be established.

The ECP fibers used in these studies were chosen due to their relative mechanical compliance and tailored electrically properties. However, several key challenges remain to facilitate extracellular electrical communication with neuronal populations using these PA–PP fibers. For instance, the relationship between conductive functionality and the secretion of acidic substances must be established, as there may be electrically functional protonation levels that are not neurotoxic. Additionally, an insulating material will be required to permit precision in extracellular stimulation, the thickness of which may be locally regulated to permit signal propagation to specific adhered neuronal populations. Interestingly, this strategy could sequester neurotoxic leachants, while maintaining sufficient protonation for PA–PP electrical functionality. Potentially, alternative pro-conductive doping schemes may be explored that may be non-toxic. In addition, smaller, more mechanically compliant ECP fibers may be desirable for transplant applications. Overall, the long-term, stable retention of electro-conductivity at physiologically relevant levels will need to be demonstrated.

In closing, this study addressed neurobiological components of developing living tissue-engineered relays on a backbone consisting of micro-scale conductive polymeric fibers, including bio-adhesive surface modifications, high density neuronal adhesion and survival, extensive neurite outgrowth and hydrogel encapsulation. Future studies will include establishing electrophysiological communication with encapsulated neuronal networks via extracellular stimulation of neuronal somata and neurites adhered to ECP fibers with real-time observation using voltage or ion-sensitive fluorescent dyes. Additionally, alternate interface modalities, such as microfluidic perfusion, may be added to increase the versatility of the microsystem and to permit the support of constructs with increased thickness or cell density (Cullen *et al* 2007c). This work is a first step toward tissue-engineered neural-electrical relays that may be transplanted into the nervous system for integration with matrix- or tissue-bound neural cells. Ideally, the physical and biochemical properties of the encapsulating hydrogel may be engineered to facilitate host axonal growth

across the hydrogel to innervate with surviving neurons adhered to the ECP backbone. At the same time, hydrogel encapsulation may result in a physical barrier to inhibit access of endogenous migratory/recruited cells to the conductive fibers. In practice, a multitude of relays may be used in parallel to create complex interface microsystems. Overall, 'wet' biohybrid neural interface microsystems generated from these components may prove useful as investigative platforms *in vitro* or as biohybrid relays *in vivo*.

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