Reflectin as a Material for Neural Stem Cell Growth

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Supporting Information

ABSTRACT: Cephalopods possess remarkable camouflage capabilities, which are enabled by their complex skin structure and sophisticated nervous system. Such unique characteristics have in turn inspired the design of novel functional materials and devices. Within this context, recent studies have focused on investigating the self-assembly, optical, and electrical properties of reflectin, a protein that plays a key role in cephalopod structural coloration. Herein, we report the discovery that reflectin constitutes an effective material for the growth of human neural stem/progenitor cells. Our findings may hold relevance both for understanding cephalopod embryogenesis and for developing improved protein-based bioelectronic devices.

KEYWORDS: reflectin, cephalopods, bioelectronics, biointerfaces, stem cells

INTRODUCTION

Cephalopods (squid, octopuses, and cuttlefish) are renowned as much for their stunning camouflage displays as for their vertebrate-like behavioral characteristics.1−6 These abilities stem from the cephalopods’ sophisticated nervous system, which has evolved to exhibit a number of anatomical and functional similarities with the nervous systems of vertebrates.4,6,11 Consequently, cephalopods have facilitated fundamental neurobiology research by furnishing seminal comparative neurophysiological model systems, including giant axons, chemical synapses, and chromatophore neuromuscular junctions.12−19 The study of cephalopods has therefore illuminated basic mechanisms of neuronal communication and greatly advanced scientific understanding of human brain function.6,10−11

Outside of neuroscience, the complex skin structure of cephalopods has served as a source of inspiration for the development of novel functional devices from both artificial20−25 and natural materials.20−29 For example, several groups have fabricated stimuli-responsive optical coatings from proteins known as reflectins, which play a crucial role in cephalopod structural coloration and possess unique self-assembly properties.26−34 Moreover, the reflectin A1 isoform from Doryteuthis (Loligo) pealeii has been shown to function as an effective proton conduction medium, enabling its use in protonic transistors.35,36 These findings have indicated that reflectins possess untapped potential as active materials not only for adaptive optics but also for bioelectronic devices.

Recently, cephalopods, and specifically the cuttlefish Sepia officinalis, have been touted as promising comparative models for ecological and evolutionary developmental biology.37−39 Within this context, the presence of both mRNA and iridescence associated with reflectin were noted during the later stages of embryogenesis in Sepia officinalis.40,41 Interestingly, reflectin was detected during developmental stages that correlate to some of the major steps of neurogenesis, precisely when brain maturation and growth occurs in Sepia officinalis embryos.40,41 These observations hinted at undiscovered roles for reflectin in cephalopod neural development and inspired us to consider the possibility that reflectin could promote neural stem cell growth.

Herein, we report the finding that the reflectin A1 isoform constitutes an effective substrate material for human neural stem/progenitor cells (hNSPCs). We first fabricate reflectin films according to standard protocols.26,35,36 We next show that these substrates support the growth of murine and human cells. We subsequently demonstrate that reflectin films facilitate the adhesion, proliferation, and differentiation of relatively difficult-to-culture hNSPCs. Our findings represent a crucial
RESULTS AND DISCUSSION

We began our studies by fabricating reflectin films on quartz or silica substrates. We first expressed a histidine-tagged Doryteuthis (Loligo) pealeii reflectin A1 isoform in E. coli. We then coated the substrates with reflectin via a modified doctor blading procedure (Figure 1), yielding films that were similar to those reported in our previous studies.26,27 The resulting reflectin-coated substrates were used directly for cell culture experiments (Figure 1).

We initially evaluated the ability of reflectin to support the growth of intrinsically adherent murine and human cells (before transitioning to a more challenging cell type). For these experiments, we specifically selected three established cell lines that are well-known to grow on varied surfaces: HEK293A (human embryonic kidney cells that are widespread in cell biology),22−24 MDA-MB-231 (human mammary cells that are employed in breast cancer research),25−27 and BV2 (murine microglial cell lines that are used for exploring the immune response of the central nervous system).48−50 The use of these established cell lines facilitated our exploratory protocol optimization.

We incubated reflectin-coated substrates with HEK293A, MDA-MB-231, or BV2 cells. After 1 day, we visualized the substrates with bright-field microscopy (Figure 2). The three cell types exhibited morphologies that were consistent with literature precedent and indicative of adhesion and/or division. Indeed, HEK293A cells grew together in islands and possessed relatively flat bodies;22−24 MDA-MB-231 cells featured granular and spindle-like bodies;45−47 and BV2 cells varied between flat spinous and small rounded bodies.48−50 These observations demonstrated that reflectin films were capable of supporting mammalian cell adhesion and portended favorably for the subsequent experiments.

We proceeded to challenge our substrates with hNSPCs, which play an essential role in brain and spinal cord development.51−53 As a general rule, stem cells are exquisitely sensitive to chemical and physical cues from their surrounding environment, making them relatively difficult to culture on arbitrary surfaces.54−60 Here, we focused our efforts on SC27 hNSPCs derived from the cerebral cortex of a post-mortem fetal brain.51,62 These cells express standard neural stem cell markers, including SOX2, nestin, and CD 133, and can potentially differentiate into three of the major brain cell types (i.e., astrocytes, neurons, and oligodendrocytes).61−65 They have also been shown to possess therapeutic potential in mouse models of Sandhoff disease.66 Furthermore, we have previously explored the adhesion, proliferation, and differentiation of SC27 hNSPCs under varied conditions on different substrates.63,64,67 Thus, SC27 hNSPCs represented a particularly advantageous choice for our studies.

We seeded reflectin-coated and uncoated substrates with undifferentiated SC27 hNSPCs and visualized them with phase contrast optical microscopy over a period of 15 days (Figure 3). For the coated substrates, we were gratified to observe that the hNSPCs were bound to the surface after 1 day. Indeed, most of the cells adopted a predominantly elongated morphology, and some of the cells displayed a rounded morphology while maintaining attachment to the substrate, as might be expected during cell division (Figure 3A). After 15 days, we found a marked >10-fold increase in the cell density, confirming division (Figure 3D). In contrast, uncoated substrates showed no hNSPC binding or growth under identical conditions over
the same time period (Figure 3D). Together, these observations indicated that reflectin coatings facilitated hNSPC attachment and subsequent proliferation on their surfaces (and supported cell viability for an extended period of time).

We next directly compared hNSPC growth on reflectin and more established materials (Figure 4). Due to their significance in both the fetal and adult central nervous systems, hNSPCs have been cultured on various substrates, including ceramics, polymers, polysaccharides, synthetic peptides, and naturally occurring proteins.54–56 For our comparison, we selected fibronectin, laminin, and poly-D-lysine. These coatings were specifically chosen because they are in the same materials class as reflectin (i.e., proteins and peptides)54–56 and have been previously used for the growth of hNSPCs.63,64,67

Phase contrast optical microscopy images of cells cultured under identical conditions on reflectin, fibronectin, laminin, and poly-D-lysine are shown in Figure 4. Here, because stem cells often produce their own extracellular matrices, which contribute to cell adhesion and proliferation over extended periods of time, we limited our analysis to the first 3 days of growth. Although hNSPCs were not always uniformly distributed on reflectin-coated substrates (Figure 4A), the cell morphologies were similar to those observed on fibronectin- and laminin-coated substrates (Figure 4B,C) but quite distinct from those observed on poly-D-lysine-coated substrates (Figure 4D). Moreover, we observed that the cell densities on reflectin, fibronectin, and laminin were comparable and much higher than on poly-D-lysine (Figure 4E). Overall, the performance of reflectin was comparable to that of common validated neural stem cell growth matrices.

We proceeded to assess the behavior of hNSPCs cultured on reflectin-coated substrates. Because stem cell/matrix interactions influence cell fate,54,60 we investigated whether reflectin affected typical physiological processes previously documented for membrane-bound proteins at the cell–substrate interface. Thus, we studied the activity of the Ca2+−permeable ion channel Piezo1, which influences stem cell differentiation via the transduction of matrix mechanical information.67 For this purpose, we utilized total internal reflection fluorescence microscopy (TIRFM), a technique that reduces intracellular background fluorescence and specifically reports on events at the cell–substrate interface (Figure 5).68,69

We visualized hNSPCs on reflectin-coated substrates with TIRFM, monitoring them in real time (Figure 5). The fluorescent Ca2+ indicator Fluo-4AM facilitated imaging of individual cells (Figure 5B), enabling us to measure spontaneous Ca2+ transients that have been previously linked to Piezo1 activity (Video S1).67 As an example, two arbitrary representative areas are marked with blue and red boxes in Figure 5B, and the corresponding spontaneous transients are shown in Figure 5C. In general, the appearance of the hNSPCs, as well as the amplitude and frequency of the associated transients (Video S1), closely resembled those found on fibronectin-coated substrates (Video S2).67 Overall, our TIRFM measurements provided additional evidence for hNSPCs exhibiting “typical” behavior and activity on reflectin.

Having validated that reflectin-coated substrates support hNSPC adhesion and proliferation, we investigated the differentiation potential of reflectin-bound neural stem cells. Thus, after 2 days of growth in proliferation media, we induced differentiation of the hNSPCs by substituting proliferation media with differentiation media. After 14 days in the
demonstrated for traditionally differentiated hNSPCs.63,64,67 We sought to detect the presence of neurons and astrocytes but not oligodendrocytes, as they are not efficiently generated via standard differentiation protocols.

Fluorescence microscopy images of two different immunostained sets of hNSPCs are shown in Figure 6. To detect astrocytes, we labeled cells for SOX2, a stem/progenitor cell marker (Figure 6B), and GFAP, a typical astrocytic marker (Figure 6C). This co-staining approach identified the SOX2-negative and GFAP-positive cells as fully differentiated astrocytes (Figure 6D). To detect neurons, we labeled cells for a combination of MAP2 and DCX, which are common neuronal markers (Figure 6F,G). This co-staining approach identified the MAP2- and DCX-positive cells as differentiated neurons (Figure 6H). Here, we note that immunostained differentiated cells grown on reflectin (Figure 6) were almost indistinguishable from those grown on laminin under identical conditions (Figure S1). For example, the percentages of hNSPCs on reflectin that transformed into astrocytes and neurons were 64.2 ± 0.6% and 3.9 ± 0.7%, respectively, and the percentages of hNSPCs on laminin that transformed into astrocytes and neurons were 67.1 ± 6.1% and 4.2 ± 0.8%, respectively. Overall, our experiments confirmed that reflectin-coated substrates were fully capable of supporting neural stem cell differentiation.

**CONCLUSION**

In summary, we have discovered that reflectin constitutes an effective material for cell growth and differentiation, as demonstrated for traditionally difficult-to-culture hNSPCs. The viability of the reflectin-bound neural stem cells was assessed with a combination of phase-contrast, bright-field, and fluorescence microscopy techniques. Overall, our studies indicate that reflectin is quite comparable to alternative neural stem cell matrix materials.

Here, we note that our experiments may be quite interesting from the perspective of developmental and cell biology. For example, reflectin’s unexpected presence during key stages of brain development in *Sepia officinalis* embryos, together with its functionality as a surface for hNSPC growth, hint that the protein may potentially play some as-yet undetermined general role in cephalopod nervous system development. Moreover, given that positively charged reflectin is similar to fibronectin and laminin, but superior to positively charged poly-d-lysine, as a cell growth substrate, its unusual amino acid sequence30–32,34 may influence stem cell binding and/or proliferation. These possibilities are exciting and certainly warrant further exploration, especially within the context of cephalopods as general comparative model systems for vertebrates.

Finally, our findings hold particular significance from the perspective of bioelectronic applications. Indeed, relatively few materials have been shown to simultaneously exhibit excellent electrical properties and support stem cell attachment, proliferation, and differentiation.54–60 Reflectin’s ability to serve as a substrate for neural stem cell growth therefore establishes the groundwork for interfacing hNSPCs with protein-based proteonic devices and raises the intriguing possibility of directly regulating neurogenesis with proteonic currents. Moreover, in contrast with some traditional neural stem cell growth materials, reflectin possesses a similar refractive index to glass, making it nearly ideal for TIRFM measurements. The protein’s combination of favorable optical and electrical properties thus opens an opportunity for the spectroelectrochemical triggering and monitoring of ion channel activity in single isolated neural stem cells. Together, these advantages portend favorably for the future of reflectin as an inherently biocompatible active layer in a diverse array of bioelectronic devices.

**EXPERIMENTAL SECTION**

**Statement of Ethics.** Informed written consent was obtained for all human subjects. All human cell research involved cells with no patient identifiers and was approved by the University of California, Irvine Institutional Review Board and the Human Stem Cell Research Oversight Committee.

**Expression, Purification, and Characterization of Reflectin.** Reflectin was prepared according to previously reported protocols.26,27,29,36 An *E. coli* codon optimized gene coding for 6X histidine-tagged reflectin A1 protein from *Doryteuthis pealeii* (Genbank: ACZS7764.1) was synthesized and cloned into the pExpress414 vector (DNA2.0). The vector was transformed into BL21(DE3) cells (Novagen). Reflectin was expressed at 37 °C using Overnight Express Instant Terrific Broth (TB) media (Novagen) supplemented with 100 μg mL⁻¹ Carbenicillin. Reflectin was completely insoluble when expressed at 37 °C and was sequestered in inclusion bodies prepared using BugBuster (Novagen) according to

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**Figure 6.** Typical fluorescence microscopy images of two separate sets of immunostained hNSPCs on reflectin-coated substrates after differentiation into (A–D) astrocytes and (E–H) neurons. The cells were co-stained with (A) the nuclei marker Hoechst, (B) the stem/progenitor cell marker SOX2, and (C) the astrocytic marker GFAP. The image corresponding to all three markers is shown in (D), where SOX2-negative/GFAP-positive cells are differentiated astrocytes. The neurons were co-stained with (E) the nuclei marker Hoechst, (F) the neuronal marker DCX, and (G) the neuronal marker MAP2. The image corresponding to all three markers is shown in (H), where MAP2-positive and DCX-positive cells are neurons.
the manufacturer’s suggested protocols. The inclusion bodies were then solubilized in denaturing buffer (pH 7.4, 50 mM sodium phosphate, 300 mM sodium chloride, 6 M guanidine hydrochloride) and purified under denaturation conditions on a HisPur Cobalt Resin gravity column (Thermo Scientific) according to the manufacturer’s protocols (elution was performed using denaturing buffer supplemented with 250 mM imidazole). The fractions containing the reflec t protein were pooled and concentrated on an Amicon Concentrator (Millipore) before being purified with high performance liquid chromatography (HPLC) on an Agilent 1260 Infinity system using a reverse phase C18 column. The gradient was evolved from 95% Buffer A: 5% Buffer B to 5% Buffer A:95% Buffer B at a flow rate of 0.5 mL min⁻¹ over 20 min (Buffer A: 99.9% water, 0.1% trifluoroacetic acid; Buffer B: 95% acetonitrile, 4.9% water, 0.1% trifluoroacetic acid). After purification, the identity of the protein was confirmed with Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel tryptic digestion, followed by mass spectrometry analysis. The pure reflec t was pooled, flash frozen in liquid nitrogen, and lyophilized. Protein concentrations and yields were quantified via the Bradford protein assay (BioRad) with BSA as the standard.

Fabrication of Reflectin-Coated Substrates. Reflectin-coated substrates were prepared according to previously reported procedures. 26,27,30,33 For bright-field microscopy experiments, reflectin films were fabricated on silica substrates (SQI), and for phase contrast optical microscopy and fluorescence microscopy experiments, reflectin films were fabricated on glass coverslips (ThermoFisher). In a typical experiment, uncoated substrates were first cleaned sequentially with Milli-Q water, acetone, and isopropanol, as well as flame-sterilized with a bunsen burner. A fresh 10–20 mg/mL reflectin solution was then prepared and filtered through sterile 0.45 and 0.22 μm filters (ThermoFisher). Subsequently, Teflon tape (McMaster-Carr) was applied to edges of the substrates to act spacer for coating. The reflectin protein solution was then cast onto the substrate in front of a plastic blade, which was translated at a constant speed across the surface to produce films. To promote water evaporation, the coating procedure was performed at 80 °C. Note that the absence of a graphene oxide adhesion layer 26,27 reduced film uniformity over large areas.

Growth of HEK, BV2, and MDA-MB-231 Cell Cultures. The HEK cells were a gift from Dr. Naoto Hoshi, the BV2 cells were a gift from Dr. Heike Wulf, and the MDA-MB-231 cells were purchased from ATCC. HEK, BV2, and MDA-MB-231 cells were plated at densities of 20,000–105,000 cells/cm². The cells were grown as adherent cultures according to known procedures 42–50 at 37 °C and under 5% CO₂ in DMEM (Life Technologies), supplemented with 10% fetal bovine serum (Gibco/Bio-Products) and 1% penicillin/streptomycin.

Growth of Adherent Human Neural Stem/Progenitor Cell Cultures. hNSPCs denoted as SC27 were procured from the National Human Neural Stem Cell Resource. 61–63 The hNSPCs were originally derived from brain subventricular zone (SVZ) tissue of a premature neonate that died shortly after birth, as previously described. 61,62 For donation of the requisite brain tissue, informed consent was obtained prior to tissue acquisition, and this process was approved by the Institutional Review Board. 61,62 In our studies, the cells were plated at densities of 40,000–80,000 cells per 18 mm coverslip. The cells were grown as adherent cultures at 37 °C and under 5% CO₂ in base proliferation media, which consisted of DMEM:F12 (Gibco/Invitrogen), 20% (v/v) BIT 9500 (bovine serum albumin, insulin, and transferrin) (Stem Cell Technologies), and 1% (v/v) antibiotic/antimycotic (penicillin, streptomycin, and amphotericin) (Gibco/Invitrogen). This media was supplemented with 40 ng/mL epidermal growth factor (EGF) (BD Biosciences), 40 ng/mL fibroblast growth factor (FGF) (BD Biosciences), and 20 ng/mL platelet-derived growth factor (PDGF-AB) (Peprotech). To induce differentiation, the base media was exchanged for the differentiation media, which consisted of a 1:1 mixture of the base media and Neurobasal media (Invitrogen) supplemented with 0.5% (v/v) B27 (Life Technologies), 20 ng/mL brain-derived neurotrophic factor (BDNF) (Peprotech), 20 ng/mL neurotrophin-3 (NT3) (Peprotech), 1% (v/v) fetal bovine serum (FBS) (Gibco/Invitrogen), 2.5 ng/mL FGF, and 0.1 μM all-trans-retinoic acid (Sigma). The cells were maintained in the differentiation media for a minimum of 14 days.

Optical Microscopy of Adherent Mammalian Cells. Bright-field microscopy images of HEK, BV2, and MDA-MB-231 cells were obtained with a Carl Zeiss Axio Imager A1 microscope in a 145 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 5 mM Hepes, pH = 7.3 buffer solution. Phase contrast optical microscopy images of hNSPCs were obtained with an Advanced Microscopy Group EVOS XL microscope or an Olympus IX71 microscope, which was outfitted with a Hamamatsu C8484 digital camera. The hNSPC images were collected in the base proliferation media. To quantify cell densities, the images were analyzed with FIJI software. 70

Fluorescence Microscopy of Immunostained Human Neural Stem/Progenitor Cells. Fluorescence microscopy images of immunostained hNSPCs were obtained with a Nikon Eclipse Ti microscope and acquired with NIS element AR3.10 software. For imaging, the differentiated hNSPCs were first fixed by treatment with 4% paraformaldehyde for 10 min. These fixed cells were then treated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, followed by blocking in 5% bovine serum albumin in PBS for 1 h. All cell nuclei were stained with Hoechst 33342 at 1:500 (2 μg/mL) in PBS for 1 min. To detect astrocytes, the cells were stained with mouse anti-GFAP (Sigma) and goat anti-SOX-2 (Santa Cruz Biotechnology) primary antibodies for 2 h at room temperature. To detect neurons, the cells were stained with mouse anti-MAP2 (Sigma) and goat anti-DCX (Santa Cruz Biotechnology) primary antibodies for 2 h at room temperature. Both neurons and astrocytes were stained with donkey antimouse Alexa-Fluor 555 (Life Technologies) and donkey anti-goat Alexa-Fluor 488 (Life Technologies) secondary antibodies for 1 h in the dark at room temperature. The primary and secondary antibodies were diluted at 1:200 in 1% bovine serum albumin in PBS solution. To compensate for a lower neuron differentiation percentage, hNSPCs were plated at densities of 80,000 cells per 18 mm coverslip for neurons and at 40,000 cells per 18 mm coverslip for astrocytes. The images were analyzed with FIJI software. 70

Total Internal Reflection Fluorescence Microscopy Imaging of Adherent Human Neural Stem/Progenitor Cells. Fluorescence microscopy images were obtained by using an Olympus IX71 microscope equipped with an Andor iXon EMCCD camera, a Melles Griot 488 nm solid-state laser, and a 1.49 NA Olympus 100x objective lens. The images were collected with an exposure time of 0.04081 s at 13.7 Hz. For Ca²⁺ imaging, the hNSPC cells were loaded with a Ca²⁺ indicator by incubation in a solution of 1 μM Fluo-4 AM in phenol red-free DMEM/F12 (Invitrogen) for 10 min at 37 °C. The cells were then washed three times and further incubated at room temperature for another 10–15 min to allow for intracellular cleavage of the AM ester. The total internal reflection fluorescence microscopy images were obtained in a 148 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 8 mM glucose, 10 mM Hepes, pH = 7.3 buffer solution. The images were analyzed with FIJI and Origin 9.1 software.

ASSOCIATED CONTENT

S Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b08717.

Fluorescence microscopy images of differentiated, immunostained hNSPCs on laminin-coated substrates (PDF)
Video S1 of an hNSPC on a reflectin-coated substrate (ZIP)
Video S2 of an hNSPC on a fibronectin-coated substrate (ZIP)
References


