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Biocompatibility of biodegradable semiconducting melanin films for nerve tissue engineering

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ABSTRACT

The advancement of tissue engineering is contingent upon the development and implementation of advanced biomaterials. Conductive polymers have demonstrated potential for use as a medium for electrical stimulation, which has shown to be beneficial in many regenerative medicine strategies including neural and cardiac tissue engineering. Melanins are naturally occurring pigments that have previously been shown to exhibit unique electrical properties. This study evaluates the potential use of melanin films as a semiconducting material for tissue engineering applications. Melanin thin films were produced by solution processing and the physical properties were characterized. Films were molecularly smooth with a roughness (R_{ms}) of 0.341 nm and a conductivity of $7.00 \pm 1.10 \times 10^{-5}$ S cm^{-1} in the hydrated state. In vitro biocompatibility was evaluated by Schwann cell attachment and growth as well as neurite extension in PC12 cells. In vivo histology was evaluated by examining the biomaterial–tissue response of melanin implants placed in close proximity to peripheral nerve tissue. Melanin thin films enhanced Schwann cell growth and neurite extension compared to collagen films in vitro. Melanin films induced an inflammation response that was comparable to silicone implants in vivo. Furthermore, melanin implants were significantly resorbed after 8 weeks. These results suggest that solution-processed melanin thin films have the potential for use as a biodegradable semiconducting biomaterial for use in tissue engineering applications.

1. Introduction

1.1. Conducting biomaterials for tissue engineering

Electrical stimulation of tissue engineering constructs has proven to be useful in promoting tissue formation for regenerative medicine applications. Electrical stimulation has been shown to be improve the behavior of cells–scaffold constructs for neurites [1], cardiac myocytes [2], and myoblasts [3]. Conducting biomaterials have been demonstrated to be potentially useful for tissue engineering because of the potential for use as a platform that supports electrical stimulation of cell–tissue constructs. Of particular interest is the application of conducting materials for use in neuronal tissue engineering because of the direct electrophysiological link between cell function and electrical stimulation. Toward this end, the processing and properties of poly(pyrrole) (PPy) have been characterized extensively as a potential platform material for both in vitro [1] and in vivo neural tissue engineering applications [4]. PPy has also been chemically modified to improve tissue response [5] and functionality [6] to broaden the spectrum of potential applications. PPy is non-biodegradable, which limits potential applications and therefore motivated the synthesis of other conducting polymers based on conjugated aromatic systems [7–9].

1.2. Melanin biopolymers

Melanins are a class of naturally occurring conductive pigments with unique physical and electrical properties [10]. Although the exact chemical structure of melanins is still not known, eumelanins, a subclass of melanins, are thought to be extended heteropolymers of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid [11]. These heteropolymers can stack to form aggregates with strong π–π interactions. The presence of these aromatic systems is thought to provide the basis for the unique observed electrical

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properties of melanins. The electrical conductivity of melanins spans a large range and is strongly dependent on the temperature, physical form, and the hydration state. Melanin films have conductivities on the order of 10^{-8} S cm^{-1} in the dehydrated state and up to 10^{-3} S cm^{-1} in the fully hydrated state [12]. Although the exact conduction mechanism is not yet known in either extreme hydration or dehydration, there is sufficient evidence that the conduction mechanism is similar to that of typical conductive organic materials [12]. Melanins are soluble in several solvents including concentrated aqueous sodium hydroxide, aqueous ammonium hydroxide, and dimethylsulfoxide (DMSO) [13]. The aforementioned properties of melanin films suggest their potential for use in tissue engineering applications including use as a biomaterial for neural regenerative applications. However, the in vitro and in vivo biocompatibility of melanin substrates has not been studied. In this report, we describe the fabrication and characterization of solution processed melanin thin films.

2. Materials and methods

2.1. Melanin thin film fabrication and characterization

All materials were used as received. Melanin solutions were made by dissolving synthetic melanin (Sigma, St. Louis, MO, USA) at concentrations between 2 and 8% (w/v) in either DMSO (Sigma) or 1 M aqueous sodium hydroxide (Sigma). Thin films of melanin were made by spin coating filtered melanin solutions on silicon dioxide substrates at 1000 RPM for 60 s. The films were then vacuumed dried at room temperature for at least 16 h. Film thickness was determined by measuring the step height of a scratch test using a stylus profilometer (KLA Tencor, San Jose, CA, USA). The film roughness was measured by atomic force microscopy (AFM) via a Nanoscope IIIa (Veeco Digital Instruments, Santa Barbara, CA, USA). Fragments of melanin films were delaminated from substrates and analyzed with an FT-IR microscope (Nikon Instruments, Melville, NY, USA). Spectra were recorded using a Nicolet Magna 550 Series II IR Spectrometer equipped with OMNIC Software using 32 scans across the wave numbers 4000–400 cm^{-1} at a resolution of 2 cm^{-1}. Atomic composition across two samples was measured using a Kratos AXIS Ultra Imaging X-ray Photoelectron Spectrometer with Delay Line Detector. The resistivity was measured by spin coating 2% (w/v) melanin in DMSO solutions on silicon oxide substrates. Electrode contacts were made by thermal evaporation of Au directly onto the surface through a shadow mask (40 nm in thickness). Samples were incubated in a 100% humid environment for at least 48 h and immediately characterized upon removal into ambient conditions. Voltage–current measurements were performed using a 2-terminal resistor technique with a Keithley 4630A semiconductor characterization system between ±10 V (Keithley Instruments, Cleveland, OH, USA) and analyzed with KITE Software (Keithley Interactive Test Environment 5.0-SP1). Resistance measurements were made across a range of channel lengths varied between 50 and 200 μm with a constant width-to-length ratio of 20.

2.2. Cell culture

In addition to melanin films, collagen-coated silicon dioxide and uncoated silicon dioxide substrates were used as control materials. Collagen-coated slides were prepared by coating slides with a dilute solution of 50 μg mL^{-1} collagen I from calf skin (Invitrogen, Carlsbad, CA, USA) in double-distilled H_2O (ddH_2O) and allowing the solvent to evaporate overnight. Melanin substrates were sterilized by UV irradiation for 15 min. Cultures of primary SCs were initiated as previously described [14]. Dissected nerves from postnatal day 1 Sprague-Dawley rat pups (Charles River Laboratories, Wilmington, MA) were collected in L-15 Leibovitz (Invitrogen). Nerves were transferred to L-15 containing 0.1% (w/v) collagenase (Invitrogen), incubated at 37 °C for 30 min, and physically disrupted by pipetting. The cells were washed once with L-15 containing 10% fetal calf serum (FCS) (Invitrogen), plated onto 10 cm diameter Primaria dishes (Fisher Scientific) in Cb-medium, which consisted of DMEM (high glucose, pyruvate) media (Invitrogen), 10% FCS, 10 ng mL^{-1} 7S NGF, 100 μg mL^{-1} streptomycin (Invitrogen), and 100 U mL^{-1} penicillin (Invitrogen). The cells were incubated overnight at 37 °C and 5% CO_2. The next day, SCs were harvested using the cold jet method [15] and plated into Primaria dishes. SCs were expanded in DMEM containing 3% FCS, 10 μM forskolin, 5 ng mL^{-1} neuregulin-1, 100 μg mL^{-1} streptomycin, and 100 U mL^{-1} penicillin. SCs were harvested using trypsin 0.025%/EDTA 0.01% (Invitrogen) and used between passage three and ten. SCs were seeded in the presence of serum at 50,000 cells cm^{-2} at 37 °C and 5% CO_2. Typically, 90% pure populations of SCs were obtained as determined by S-100 expression. Attachment of SCs was characterized at 24 h via microscopy via a Zeiss AxioVert 200 M microscope (Carl Zeiss, Thornwood, NY, USA) equipped with AxioVision software. PC12 cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 2.5% fetal bovine serum, 1% horse serum (ATCC), and 1% penicillin–streptomycin. PC12 cells were seeded at initial densities of approximately 50,000 cells cm^{-2}. After 24 h of attachment, murine nerve growth factor (NGF; Invitrogen) was incorporated into medium at concentrations of 50 ng mL^{-1}. Medium was exchanged every other day and neurite lengths were characterized at appropriate time points via light microscopy.

2.3. In vivo biocompatibility study

The prospective use of melanin as a biomaterial for neurological applications suggested that this material would be used in intimate contact with nerves. This provided the motivation for studying the tissue–biomaterial response of melanin with the sciatic nerve. Silicone was chosen as a control material since it generates a mild, short-term immune response [16] and has been used as a biomaterial for peripheral nerve reconstruction in previous studies [17]. Melanin samples were prepared by drop casting 100 μL of 8% (w/v) melanin in DMSO solution onto smooth PDMS substrates. DMSO was removed by applying vacuum at room temperature. This resulted in melanin slabs approximately 100 μm in thickness (T) and 5 mm in diameter (D). Medical grade silicone slabs of approximately 0.5 × 3 mm ($T \times D$) were used as a control material. All implants were sterilized and disinfected for 20 min under UV and incubated in 0.9% saline prior to implantation. Slabs were implanted into seven-week-old female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA). Four female Lewis rats (Charles River Laboratories, Wilmington, MA), weighing approximately 200 g, had access to water and food *ad libitum*. Animals were cared for according to the protocols of the Committee on Animal Care of MIT in conformity with the NIH guidelines (NIH publication #85-23, revised 1985) [11]. The animals were anaesthetized using continuous 2% isoflurane/O_2 inhalation. Under sterile conditions and external body warming, the sciatic nerve on the left side was approached through a semitendinosus-biceps femoris muscle splitting approach. The sciatic nerve was exposed, the epineurium opened and the implants

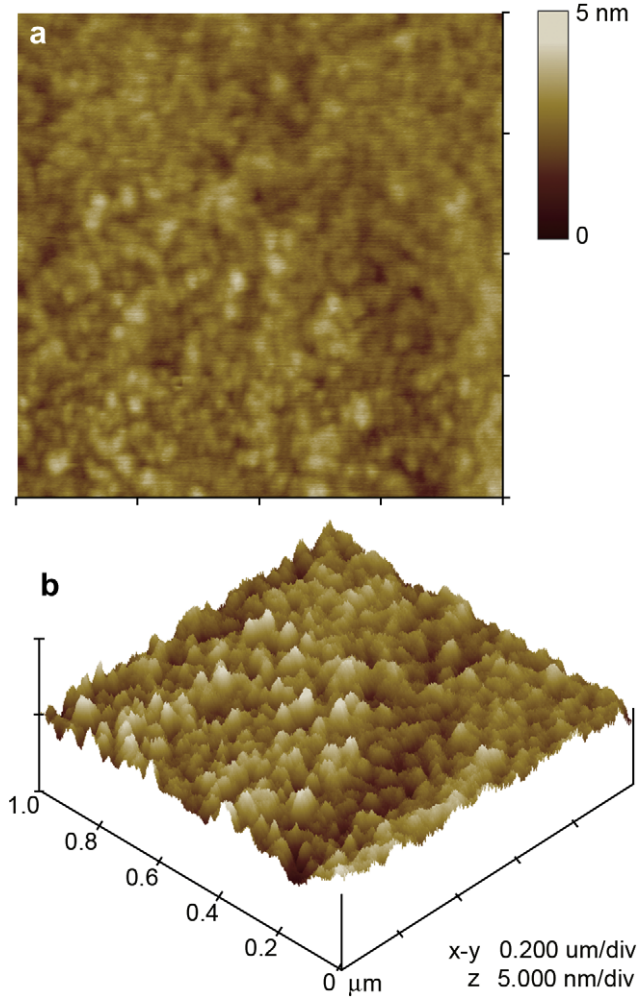

Fig. 1. AFM characterization of melanin films derived from DMSO solvent. The mean-squared roughness (R_{ms}) was calculated to be 0.341 nm.