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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 $7.00 \pm 1.10 \times 10^{-5} \text{ S cm}^{-1}$ 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.

Keywords

Electroactive polymer; Biocompatibility; Nerve tissue engineering

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 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 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 $10^{-8} \text{ S cm}^{-1}$ in the dehydrated state and up to $10^{-3} \text{ S cm}^{-1}$ 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 $\mu\text{g mL}^{-1}$ collagen I from calf skin (Invitrogen, Carlsbad, CA, USA) in double-distilled H_2O (dd H_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 Cbmedium, which consisted of DMEM (high glucose, pyruvate) media (Invitrogen), 10% FCS, 10 ng mL^{-1} 7S NGF, 100 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ 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* × *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/O₂ 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 were placed on top of the sciatic nerve, using an operating microscope. The muscle layer was then closed using 4-0 Vicryl sutures, and the skin was closed using staples. Rats were sacrificed at pre-determined time points of 1 and 8 weeks post-implantation. Tissue samples of intact implants of approximate dimensions of 15 mm × 15 mm were harvested *en bloc* for histology. The samples were fixed in Accustain (Sigma) for 24 h and embedded in paraffin after a series of dehydration steps in ethanol and xylene, and stained appropriately. Briefly, sequential sections (8–15 µm) were stained with hematoxylin and eosin (H&E) and Masson's trichrome stain to determine fibrous capsule morphology. Histology images were recorded with a Nikon CCD Camera attached to a microscope stage.

2.4. Statistics

All graphical and tabulated data displayed as mean ± S.D. unless otherwise noted. At least 200 neurite lengths were characterized across two independent experiments. SC densities were determined by calculating cell densities in ten representative images in four samples across two independent experiments. One-way and two-way ANOVA results were performed with Kruskal–Wallis and Bonferroni post-tests, respectively, where appropriate using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA). Significance levels were set at **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

3. Results

3.1. Chemical and physical properties of solution processed melanin thin films

Melanin thin films solution processed from DMSO had average thickness of 160.2 nm as determined by profilometry with an average surface roughness (R_{ms}) of 0.341 nm as measured by AFM (Fig. 1). The micron scale morphology of melanin films processed using DMSO was significantly different than films processed using sodium hydroxide solutions. Melanin films spin coated from DMSO solutions exhibited molecular scale smoothness and uniformity over large areas. This lies in stark contrast to melanin films spin coated from aqueous sodium hydroxide, which featured large, dendritic structures (Supplementary Figure 1). The thickness of the films could be tuned by changing the concentration of the solution and the spin coating speed (Supplementary Figure 2).

Melanins consist of two classes of compounds; eumelanins and pheomelanins. Although the building blocks of these two classes are primarily known [11], the exact chemical compositions of eumelanins and pheomelanins have yet to be determined. Melanin films were characterized via FT-IR (Fig. 2). FT-IR transmittance and XPS data suggest the presence of the following functional groups (Tables 1 and 2); alkenes, carboxylic acids, primary amines, and secondary amines. In addition to morphology and chemical composition, the electrical properties of melanin thin films were characterized. The degree of hydration is known to have a dramatic effect on the conduction of both melanin films and pellets [18]. Hence, it is imperative that the resistivity of the films should be calculated in the hydration state that most accurately resembles the environment of the final intended application. In the case of utilizing melanin films for neural tissue engineering applications, we can assume that these films will be immersed in aqueous medium and therefore be fully hydrated. The film thickness was measured to be 22.5 ± 3.5 nm by profilometry. Spin coated melanin films hydrated in 100% humidity were semiconductive with conductivities measured to be $7.00 \pm 1.10 \times 10^{-5}$ S cm⁻¹. There was no significant variation or trend in conductivity as a function of channel length, which suggests that the contact resistance is negligible.

3.2. In vitro biocompatibility

In vitro biocompatibility was assessed by examining the potential for melanin films to support the growth and attachment of two cell types that relevant for neural tissue engineering applications; SCs and PC12 cells, a rat pheochromocytoma cell line that responds to NGF by extending neurites. Melanin films supported the attachment of both SCs and PC12 cells. SCs cultured on melanin thin films exhibited a more spindle-like morphology when compared to SCs cultured on collagen-coated glass and uncoated glass (Fig. 3). These morphological observations suggest that SCs cultured on melanin thin films exhibit a more activated phenotype than SCs cultured on collagen-coated glass and uncoated glass. SC proliferation was also enhanced on melanin substrates compared to both collagen and uncoated substrates as measured by increased cell densities at 5 and 7 d as determined by two-way ANOVA ($***p < 0.001$; Fig. 4). The cell density of SCs cultured on collagen substrates, which served as the positive control, was higher compared to uncoated glass substrates at 5 d ($***p < 0.001$). The cell density of SCs cultured on glass substrates at later

time points was observed to decrease. This can likely be attributed to the loss of cells during medium exchanges due to poor cell adhesion. PC12 cells cultured on melanin thin films were able to attach to the substrate and extend neurites in the presence of soluble NGF (Fig. 5). The distribution of neurite extension in PC12 cells cultured on melanin was larger than those of PC12 cells cultured on collagen-coated substrates at 1 d (** $p < 0.01$), 5 d (** $p < 0.001$), and 7 d (** $p < 0.001$; Table 3; Fig. 6).

3.3. In vivo biocompatibility

The in vivo tissue response of the peripheral nerve and surrounding tissues to melanin implants appeared to be qualitatively similar to that of silicone implants (Fig. 7). Fibrous capsule formation and acute inflammation were observed in the area immediately surrounding both implants at 1 week post-implantation. Furthermore, the morphology of the nerve was not affected by either the silicone or the melanin implants at 1 and 8 weeks post-implantation, as assessed by histological evaluation. There were several additional observations of note. First, the melanin implant was mechanically fragile and, as a result, fractured into many fragments after implantation. These fragments were visible at the 1-week time point. This is likely due to local mechanical perturbation. The acute inflammation response was evident by the presence of fibrous tissue and macrophages which surrounded these melanin fragments. Several incidences of giant cell formation were also found to be in close proximity to these melanin fragments. Week 8 time points indicated a chronic inflammation response in the cases of silicone implants. This was noted by the presence of a reduced fibrous capsule thickness surrounding the implant. The silicone implant itself was not significantly altered, which is to be expected as no degradation was anticipated. This lies in contrast to the melanin implants in which the material had almost completely degraded (Fig. 7, inset). Only small fragments of the melanin remained after 8 weeks. There was fibrous tissue present at the implant site as well. This fibrous tissue surrounding the implants had mostly subsided in parallel with the degrading material.

4. Discussion

Melanins are an intriguing naturally occurring class of compounds that have potential for a wide range of biomedical applications including tissue engineering. Yet, the biocompatibility of these materials has not been adequately characterized. The goal of this study was to provide a biocompatibility study to substantiate the potential role of melanins as a biodegradable material with semiconducting properties for potential use in scaffolds for tissue engineering.

4.1. Melanin films are suitable as a tissue engineering biomaterial

The strong influence of hydration in the electrical conductivity of melanin films limits the use of melanin as a material for many device applications. However, the use of melanin films for tissue engineering would assume that these films would be under fully hydrated conditions in vitro or in vivo. Furthermore, melanin films in the fully hydrated state exhibit the highest conductivities, which would further enable the use of melanin as an electrically active biopolymer for use in tissue engineering. The conductivity of melanin films in the in vivo environment is also hypothesized to be increased due to the presence of doping through

native ionic species such as sodium and potassium. The conductivities of melanin films in this study ($7.00 \times 10^{-5} \text{ S cm}^{-1}$) were significantly lower than other conducting polymers including PPy and polyaniline, which exhibit conductivities up to 10 S cm^{-1} [5] and 1 S cm^{-1} [19], respectively. However, this value of conductivity is on the same order as other synthetic biodegradable conducting polymers [8]. The rapid growth and spreading of SCs and increased neurite growth of PC12 cells cultured on melanin thin films confirm that melanin films elicit a comparable cell–biomaterial interaction as collagen films, the positive control selected in both cases. This observation suggests that melanin exhibits suitable surface properties that enable melanin to be potentially used as a biomaterial for tissue engineering scaffolds. Furthermore, these conductive films were observed to be biodegradable in vivo. Hence, we propose that melanin is convenient material that could be added to the library of currently available biodegradable conducting polymers [8,9]. Melanins also have significant advantages in that they are commercially available and are hypothesized to degrade into naturally occurring molecules including dopamine, tyrosine, and their derivatives [20,21]. However, the detailed mechanisms of degradation have not been elucidated to date. Furthermore, the precise mode of degradation in vivo is also unknown. We hypothesize that the mechanical properties of the melanin implant directly influenced the degradation rate in vivo. Solution-processed melanin films were mechanically rigid. The rigid properties of melanin implants led to fracture of the implant after insertion into the host. The presence of small melanin particulates could have allowed immediate uptake by macrophages and giant cells, both of which were observed at 1 and 8 weeks (Fig. 7). Further intracellular degradation of the melanin particles could then take place within these cells. Therefore, it is important to make a distinction between gross disintegration of the melanin implant and complete degradation of the melanin heteropolymers into monomers. In this study, it is noted that the gross erosion of the melanin implant was almost fully completed by 8 weeks. In terms of utilizing melanin as a biomaterial for a regenerative medicine implant such as a scaffold, it is appropriate to consider this to be the appropriate time scale for degradation. However, further intracellular chemical breakdown could still be occurring well after this time period.

4.2. Rapid, scalable fabrication of melanin substrates

The soluble processing of melanin films is an enabling property that allows for flexibility in a tissue engineering platform material. First, producing melanin solutions using a relatively benign organic solvent enables the fabrication of thin films with precisely controlled thickness with little concern for potential cytotoxicity. Although the films in this study were approximately 160 nm in thickness, films as thin as 4 nm have been produced by simply reducing the concentration and increasing the spin rate. Second, solution processing could enable the potential rapid patterning of substrates using soft lithography methods [22] such as capillary force lithography [23] and microcontact printing, which has been used for the patterning of proteins [24]. The potential applications of melanin thin films are intriguing because of the following material properties; 1) ease of processing, 2) semi conductive electronic properties, and 3) in vitro and in vivo biocompatibility as well as in vivo biodegradation. The use of melanin as a biomaterial in tissue engineering scaffolds and devices could further enable the use of electrical stimulation in tissue engineering constructs, which is known to be a promising avenue for stimulating the formation of in vivo

architecture in vitro. The combination of unique physical properties, biocompatibility, and degradation suggests that melanin is a promising material for applications in tissue engineering and regenerative medicine.

5. Conclusions

This work has characterized the physical, chemical and electrical properties of thin films including biocompatibility in vitro and in vivo. Melanin films were solution processed into molecularly smooth films with conductivities of $7.00 \pm 1.10 \times 10^{-5} \text{ S cm}^{-1}$. Melanin substrates enhanced proliferation in SCs and neurite extension in PC12 cells in vitro. Melanin implants introduced in close proximity to peripheral nerves in rats induced a foreign body response and a benign effect on nerve tissue that was qualitatively comparable to silicone. Furthermore, melanin implants were observed to have been almost fully degraded after 8 weeks. These results suggest that solution-processed melanin is a biodegradable semiconducting material with good biocompatibility in nerve tissue that could have potential utility as a biomaterial for tissue engineering applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Figures with essential colour discrimination. Fig. 7 of this article is difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.02.018.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the on-line version, at doi: 10.1016/j.biomaterials.2009.02.018.

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

Fig. 2. FT-IR characterization of synthetic melanin. The transmission spectrum suggests the presence of several functional groups including alkenes and amines (see Table 1).

Fig. 3. Representative bright field micrographs indicating Schwann cell morphology and attachment after 7 d on (a) melanin, (b) collagen, and (c) uncoated glass substrates. Scale bar represents 100 μm .

Fig. 4. Schwann cell growth dynamics. SCs cultured on melanin substrates grew more rapidly compared to SCs cultured on collagen (***) or uncoated substrates (***) (*** $p < 0.001$). The low cell densities observed in the uncoated condition at 5 and 7 d can likely be attributed to the low adhesion of cells to glass substrates.

Fig. 5.

Representative micrographs of neurite growth from PC12 cells cultured for up to 7 d in the presence of nerve growth factor (NGF) after on uncoated, collagen-coated, and melanin substrates. Although the neurite lengths of PC12 cells cultured on melanin substrates were observed to be larger than those of PC12 cells cultured on collagen (positive control) and uncoated (negative control) substrates, the growth rate of PC12 cells was observed to be more rapid on collagen substrates. Scale bar represents 200 μm .

Fig. 6.

Quantification of neurite lengths of PC12 cells cultured on various substrates. In general, neurite lengths of PC12 cells cultured on melanin substrates were statistically larger than those in PC12 cells cultured on collagen substrates. Neurites in PC12 cells cultured on collagen substrates were also larger than those in PC12 cells cultured on uncoated glass substrates (see Table 3). Neurite lengths in X-axis labels indicate the maximum value in each bin.

Fig. 7.

In vivo response of nerve to melanin implants. Acute inflammation was present in the area immediately surrounding both melanin and silicone implants as assessed by the presence of fibrous capsules at 1-week time points. Silicone implants remained in tact, but melanin implants appeared to fracture under the dynamic mechanically active in vivo environment. The nerve appeared to be unaffected by the presence of both the melanin and silicone implants as assessed by histological examination. The non-degraded silicone implant was contained in a mature fibrous capsule at 8 weeks. The majority of the melanin implant appeared to be resorbed as assessed by macroscopic evaluation. There were small fragments of the melanin implant still visible (inset). Scale bars represent 200 μm for low magnification images and 20 μm for high magnification inset images.

Table 1

FT-IR absorption peaks of melanin thin films.

Wavenumber (cm ⁻¹)	Broad/narrow	Functional group
3410	Broad	Alcohol or carboxylic acid (O–H stretch)
3050	Narrow	Alkenes (C–H stretch)
2915	Narrow	Alkanes (C–H stretch)
2850	Narrow	Alkanes (C–H stretch)
1713	Narrow	Ketone, carboxylic acid (C=O stretch)
1635	Narrow	Primary amine (NH ₂ scissors)
1612	Narrow	Primary amine (N–H bend)
1490	Narrow	Secondary amine
674	Narrow	Primary amine (NH ₂ scissors)

Table 2

Elemental composition of melanin thin films.

Basis	C (1s)	O (1s)	N (1s)
Atom (%)	71.0	23.5	5.5
Mass (%)	65.3	28.8	5.9

Table 3

Summary of Dunn's multiple comparison tests for neurite lengths in PC12s cultured on melanin, collagen, and untreated glass substrates.

Comparison	1 d	3 d	5 d	7 d
Melanin vs. collagen	***	ns	***	***
Melanin vs. uncoated	**	**	***	***
Collagen vs. uncoated	ns	**	**	*

Significance levels were set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Comparisons that are not statistically significant are denoted by ns.