



Bioactive diamond scaffolds support neuronal survival and axonal growth

R J F Sørensen^a, Nicolas Bertram^{a,b,1}, Ugne Dubonyte^{a,c,1}, Bob A Hersbach^c, Alison Salvador^c, Anpan Han^b, Agnete Kirkeby^{a,c}, Rune W Berg^{a,**}, Jaspreet Kaur^{a,d,*}

^a Department of Neuroscience, University of Copenhagen, Blegdamsvej 3, Copenhagen N, 2200, Denmark

^b Department of Civil and Mechanical Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

^c Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), University of Copenhagen, Blegdamsvej 3, Copenhagen N, 2200, Denmark

^d Center for Translational Neuromedicine, University of Copenhagen, Blegdamsvej 3, Copenhagen N, 2200, Denmark

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ABSTRACT

Injury to the central nervous system (CNS) can have devastating consequences for the individual, and strategies to promote endogenous axonal regeneration may be a promising future therapeutic avenue. In the case of spinal cord injury, one approach is to generate a scaffold-bridge across the injury site, through which the neuronal axons can grow and reconnect. Inspired by the various properties of diamond, including its chemical inertness, we proposed a strategy in which synthetic diamond scaffolds were coated with proteins with beneficial properties to promote biocompatibility of the scaffolds towards neurons. Here, we demonstrated that bare, non-coated diamond scaffolds, when terminated with either oxygen or hydrogen, were unable to adhere to the human embryonic stem cell-derived interneurons in culture. In contrast, oxygen terminated protein-coated scaffolds (i.e., bioactive diamond scaffold) efficiently enabled neuronal attachment and supported the survival, migration, and neurite elongation across an induced injury gap in culture. Further, hydrogen terminated bioactive scaffolds also promoted cell adhesion, migration, and neurite elongation upon injury, but not as efficiently as oxygen-terminated bioactive scaffolds. With this data we suggest that bioactive synthetic diamond scaffolds could provide a valuable tool for future therapeutic strategies in the context of CNS injuries.

1. Introduction

Central nervous system (CNS) injuries occur each year worldwide with incidences ranging from 27 to 69 million cases of traumatic brain injury (TBI) [1,2] and more than 0.9 million cases of spinal cord injury (SCI) [1]. Ample progress has been made in understanding the pathophysiological cascades that occur after the CNS injury, such as excitotoxicity [3,4], dysmetabolic conditions [5,6] and activation of inflammatory pathways [7,8]. However, the limited regenerative capacity of the CNS poses a potential challenge for effective treatment strategies. Recently, the scaffold engineering approach has gained significant attention by facilitating neuronal regeneration. Carbon-based scaffolds such as carbon nanotubes [9] and graphene [10], polymer scaffolds [11] in rats, peptide [12], hydrogel [13] and carbon [8] scaffolds in mice, and collagen scaffolds in human embryonic stem cells (hESCs) [14] have been tested but promoted only limited axonal regeneration after the

TBI [11,13] and SCI [11,14]. Hence, there is a pressing need for alternative scaffolding material to support the injured CNS tissue mechanically, encourage abundance of neurite regeneration, and thus replace the damaged tissue of the injury. One such material is nanopolycrystalline diamond, which has inspired hope in regenerative medicine and tissue engineering due to its unique structural, electrical, and chemical properties [15]. Recent advancements have enabled the fabrication of diamond structures with unique properties, such as electrical conductance and therefore recording and stimulation capacities [16]. Diamond is also biocompatible and chemically stable, making it an ideal material for use in the CNS for the development of neural prostheses and interfaces [15,17–20]. Compared with metallic [21] or silicon [22] implants, diamond-coated substrates exhibit enhanced structural and functional connections with bone [21–23]. Moreover, diamond-coated microchips for the retina have shown promising in vivo and in vitro results [24]. These microchips were grown on silicon substrates under

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* Correspondence author at. Center for Translational Neuromedicine and Department of Neuroscience, University of Copenhagen, 2200, Denmark.

** Department of Neuroscience, University of Copenhagen, 2200, Denmark.

E-mail addresses: jakob.fleng@sund.ku.dk (R.J.F. Sørensen), bertram.nic@gmail.com (N. Bertram), dubonyte@sund.ku.dk (U. Dubonyte), bob.hersbach@sund.ku.dk (B.A. Hersbach), alison.salvador@sund.ku.dk (A. Salvador), anph2@dtu.dk (A. Han), agnete.kirkeby@sund.ku.dk (A. Kirkeby), runeb@sund.ku.dk (R.W. Berg), jaspreet.kaur@sund.ku.dk (J. Kaur).

¹ The authors share an equal contribution

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low percentage of hydrogen coating (1 %) at 400°C. These optimal parameters promoted biostability, bio-inertness and no inflammatory response when tested for 6 months in rabbit. Further, three-dimensional retinal implant with a distant ground grid [25] to enhance spatial resolution also showed an increased number of bipolar neurons, which makes them candidates for implantable medical devices. In these cases, it is crucial to choose a material with high injection limits such as diamond which limits the risk of tissue and scaffold degeneration that may occur due to the injection of increased charge densities. However, boron-doped nanocrystalline diamond (where boron makes diamond electrically conductive) has been shown to induce only a mild inflammatory response one month after subcutaneous implantation in rats [19]. This mild inflammation might be due to; 1) the high doping concentration of boron necessary for high conductivity that can be slightly deleterious for the biological tissue, and 2) its surface can be less cell adhesive due to hydrogen-termination which provided hydrophobic surface chemistry [26].

Here, the diamond scaffolds we used were grown as ultrathin films of ~2 µm on surfaces. After material processing in a clean room, the final cantilevers become flexible and thus can integrate well into biological structures. We hypothesize that these diamond scaffolds can be beneficial for regeneration, especially if the surface is terminated with either hydrogen (from the air) or oxygen (from the ozone) and various protein coatings (to make them bioactive). Hence, these scaffolds could function as regenerative bioactive scaffolds for promoting neuronal recruitment, adhesion, and regrowth. To test this hypothesis, we used medial ganglionic eminence (MGE) interneurons generated from hESCs as an in vitro model for human neuronal biocompatibility, injury, and regeneration. Scaffolds treated under various conditions were introduced into neuronal cultures and mechanically injured cultures. Bare scaffolds were not able to recruit interneurons. In contrast, the placement of bioactive scaffolds at the injury site promoted substantial recruitment, adhesion, and ordered growth of most neurons. However, neurites were grown along the scaffold cantilevers, and there was greater favorability for oxygen-terminated scaffolds than for injured interneurons. This in vitro model demonstrated that bioactive diamond scaffolds have the potential to guide axonal outgrowth following CNS injury in humans.

2. Methods

2.1. Surface characterization of diamond scaffolds

The surface of the diamond scaffolds obtained from the silicon (Si) wafer was characterized using Bruker Dimension Icon atomic force microscope contact mode, where a scan size of 4×4 µm² was used and 256 lines were scanned at a rate of 1 Hz. Two samples from each condition were scanned, and the root-mean-square roughness (Rq) was averaged for each condition using Gwyddion software.

2.2. Surface functionalization of diamond scaffolds

To introduce functional groups (Supplementary Table 1) onto the diamond scaffold surface, oxidation was carried out using an ultraviolet (UV) chamber (ELG100S, Dinies Technologies GmbH, Germany). The chamber was equipped with 11-Watt UV lamps with a wavelength of 215 nm that generated ozone (O₃). The scaffolds were irradiated with 15 mW/cm² UV photons (in atmospheric air) at a 22 mm distance.

2.3. Protein and axonal growth factor coating of the scaffolds

Hydrogen containing functional groups on the scaffold surface were adapted from the air resulting in creation of hydrogen-terminated diamond scaffolds. Whereas oxygen containing functional groups were added by UV irradiation of scaffolds for 4 hours, which increased the number of negative charges on the scaffold surface, resulting in oxygen-terminated diamond scaffolds. According to the previous reports, ini-

tial coating of polylysine can highly enhance cell adhesion and viability [27–30]. Therefore, hydrogen and oxygen-terminated scaffolds were initially coated with 10 µg/cm² poly-D-lysine (PDL) by incubating them in a 1 mL solution of PDL at a concentration of 0.1 mg/mL overnight at 4°C. During the incubation step, positive charges were deposited on the scaffold surface due to predominant positive charge of PDL at physiological pH [31]. Subsequently, the scaffold surface was coated with GDNF at a concentration of 5.25 ng/cm² using 1 mL of 20 ng/mL, anti-NGO-A at 2.63 µg/cm² using 0.5 mL of 20 µg/mL, FGF2 at 40 ng/cm² using 1.46 mL of 20 ng/mL, and laminin-521 at 5.479 µg/cm² using 0.5 mL of 40 µg/mL solution. This coating was applied collectively to a Costar® 12-well clear TC-treated multiple-well plate, with each well having a surface area of 3.65 cm². The surface area of each diamond scaffold was calculated to be 22.5 mm², with the protein layer assumed to be 3.5 nm thick [32].

2.4. X-ray photoelectron spectroscopy

1 cm² nanopolycrystalline diamond (NPCD) segments (as described in the result section 3.1, fabrication of the nanopolycrystalline diamond scaffold) underwent surface functionalization. The control groups were only exposed to Dulbecco's phosphate-buffered saline (DPBS). Subsequently, the samples were analyzed using an ESCALAB 250 Xi XPS. XPS binding energies were identified from the NIST Standard Reference Database and manually confirmed by Avantage Software.

2.5. Preparation of culture media for hESCs

N2 medium: To prepare 50 mL of N2 medium, 24.5 mL of DMEM/F12 and 24.5 mL of MACS® Neuro Medium were mixed. The mixture was supplemented with 0.5 mL of N-2 supplement at a final concentration of 1 % (vol/vol). Furthermore, 0.25 mL of L-glutamine was added to the mixture at a final concentration of 2 mM. Finally, 100 µL of penicillin-streptomycin (P/S) was added to the medium, resulting in a final concentration of 0.2 % (vol/vol).

B27 Medium: To prepare 50 mL of B27 medium, 48 mL of MACS® Neuro Medium was supplemented with 1 mL of B-27 supplement without vitamin A, resulting in a concentration of 2 % (vol/vol). The medium was further supplemented with 0.5 mL of L-glutamine, resulting in a concentration of 2 mM. Finally, 100 µL of penicillin-streptomycin (P/S) was added to the medium at a final concentration of 0.2 % (vol/vol).

Wash Medium: To prepare a volume of 100 mL of wash medium, 90 mL of DMEM/F12 was supplemented with 10 mL of 10 % Knock Out Serum Replacement, resulting in a final concentration of 1 %.

2.6. Differentiation of hESCs into MGE-derived interneurons

hESCs (cell source: RC17 line, hPSCreg RGe021-A) (Supplementary Table 2) were cultured and derived into interneurons in vitro using the following protocol adapted from Nolbrant et al [33]. The hESCs were first maintained in iPS-Brew medium on 1 µg of Lam-521 coated culture plates. When the culture was confluent, the cells were passaged with 0.5 mM EDTA and seeded at a density of 2,500 cells/cm² with 10 µM of the ROCK inhibitor Y-27632. Three days prior to the start of differentiation, the cells were passaged such that the cultures covered 70–90 % of the well area at the time of differentiation.

Differentiation of hESCs into medial ganglionic eminence (MGE)-derived interneurons was initiated by seeding cells at a density of 25,000 cells/cm² on day 0 onto Lam-111 (2 µg/mL) in N2 differentiation medium (DMEM/F12:Neurobasal with 1 % N2 supplement) supplemented with Y-27632 (10 µM), SB431542 (10 µM), Noggin (100 ng/mL), and XAV939 (3 µM). On day 2, the medium was changed to N2 medium supplemented with SB431542 (10 µM), Noggin (100 ng/mL), and XAV939 (3 µM). A total of 250 µL was added to each 1 cm² of the well. On day 3, 200 ng/mL of Shh-C24II (R&D Systems) was added to the cells. On day 4 and day 7, the medium was replaced

with new N2 medium supplemented with SB431542 (10 μ M), Noggin (100 ng/ml), XAV939 (3 μ M), and 200 ng/ml of Shh-C24II. Then, 300–350 μ l of the medium was added per cm^2 . On day 9, the old medium was replaced with new N2 medium supplemented with 200 ng/ml of Shh-C24II (400 μ l/ cm^2). On day 11, the cells were dissociated with Accutase (75 μ l/ cm^2) and reseeded onto Lam-111 at 800,000 cells/ cm^2 in B27 medium (neurobasal with 2% B27 supplement) supplemented with Y-27632 (10 μ M), BDNF (20 ng/ml), ascorbic acid (0.2 mM), and 200 ng/ml of Shh-C24II. On day 14, the medium was replaced with fresh B27 medium supplemented with BDNF (20 ng/ml) and AA (0.2 mM). On day 16, the cells were dissociated with Accutase and reseeded onto Lam-521 (2 μ g/ cm^2) at 270,000 cells/ cm^2 in B27 medium supplemented with Y-27632 (10 μ M), BDNF (20 ng/ml), AA (0.2 mM), db-cAMP (500 μ M), and DAPT (1 μ M). From days 18–42, the medium was changed every 2–3 days by replacing the medium with fresh B27 medium supplemented with BDNF (20 ng/ml), AA (0.2 mM), db-cAMP (500 μ M), and DAPT (1 μ M) at a volume of 500 μ l per cm^2 . Starting from day 25, only 75 % of the medium was replaced to minimize the risk of cell detachment. Information about all the factors and media used here is provided in Supplementary Table 2.

Two batches of MGE-derived interneurons were prepared. The first batch was used for immunocytochemistry (ICC), and the second batch was used for scanning electron microscopy (SEM). On day 35, the cells reached terminal maturity, and the diamond scaffolds were introduced to the cell cultures, and the cultures were grown for 45 days.

2.7. Cell viability assay

Cell viability upon introducing diamond scaffolds was measured as follows with the following conditions (Supplementary Fig. 1): 1) control (cultures without any treatment), 2) H+D (cultures with hydrogen-terminated diamond scaffolds), H+BioD (cultures with hydrogen-terminated and protein coated scaffolds), O+D (cultures with oxygen-terminated scaffolds) and O+BioD (cultures with oxygen-terminated and protein coated scaffolds). Cells were differentiated as described in section 2.6, and diamond scaffolds of the indicated conditions were added to the culture at D35. After 5 days, cells were washed with DPBS and incubated in NB21 medium supplemented with Hoechst (5 μ g/ml) and Propidium Iodide (PI) (5 μ g/ml) for 30 minutes at 37°C and 5% CO_2 . After incubation, cells were washed twice with DPBS and cultured in fresh NB21 medium for imaging. Imaging was performed using a Leica AF6000 Widefield Fluorescence Microscope in a closed gas chamber at 5% CO_2 . Cell viability was calculated by determining the number of live cells (Hoechst positive) and dead cells (PI positive), summing these values, and dividing the number of dead cells by the total cell count. Live and dead cells were quantified using QuPath (v0.5) and StarDist (v0.4.4).

2.8. qRT-PCR

The gene expression profile of the cells differentiated towards neural lineages, were analyzed through qRT-PCR. At the indicated time points, cells were collected in RLT Buffer (from RNeasy Plus Micro kit, Qiagen 74034) with β -mercapto-ethanol (Sigma-Aldrich 63689) and stored at -80°C. For RNA isolation, all samples were processed on a QIAcube robot using the RNeasy Plus Micro kit (Qiagen 74034) and then quantified using NanoDrop 2000 (Thermo Fisher) verifying that all the samples had a 260nm/280 nm ratio close to 2. cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific K1642) with 1000 ng of input RNA and diluted in EB buffer (Qiagen 19086). RT-PCR was performed using an LIDOT Liquid Handling System (Dipendix), where 0.4 μ l cDNA was mixed with 1.6 μ l of forward and reverse primer (as given in Table 1) mix (0.96 μ M) and 2 μ l SYBR Green 2x Master Mix (Roche 048873520). RT-PCR was run on a Light-Cycler 480 II (Roche 05015243001) with a custom program of 40 cycles at 60°C. Quantification was performed using the delta delta ct method

Table 1
qRT-PCR primers.

Gene	Full name gene	Primer sequence (forward/reverse)
ACTB	Actin beta	CCTTGCACATGCCGGAG/ GCACAGAGCCTCGCCTT
ARX	Aristaless related homeobox	CCTGAGCATTCTCGGAGCG/ TGGAAAAGAGCCTGCCGAATGCC
FOXP1	Forkhead box G1	TGGCCCATGTGCGCCTTCCT/ GCCGACGTGGTGGCGTGTGTA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TTGAGGTCAATGAAGGGGTC/ GAAGGTGAAGGTGCGAGTCA
LHX6	LIM Homeobox 6	AGGCAAGAACATCTGCTCCAG/ GCCAGATGAGGTGTGTACCTT
ASCL1	Achaete-scute amily bHLH transcription factor 1	CTAAAGATGCAGGTGTGTGCG/ GGAGCTTCTCGACTTCACCA
NKX 2-1	NK2 homeobox 1	AGGGCGGGGCACAGATTGGA/ GCTGGCAGAGTGTGCCAGA

using the RC17 pluripotent cell line as a reference. For normalization two housekeeping genes were used, ACTB and GAPDH. Gene expression data were analyzed using both parametric and non-parametric statistical tests. For each gene, the data were tested for normality using the Shapiro-Wilk test. If the data were normally distributed, differences between timepoints were assessed using an unpaired two-sample t-test. If the data were not normally distributed, a Mann-Whitney U test (a non-parametric alternative to the t-test) was performed instead. Statistical significance was set at $p < 0.05$. All analyses were conducted using Python (version 3.11.3) with the SciPy (1.10.1) and Seaborn libraries (0.13.2).

2.9. Immunocytochemistry

Immunocytochemistry was performed to evaluate the expression of specific neuronal markers on cells cultured on the diamond scaffolds. Prior to fixation on day 16 and 42, cells were washed once in DPBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$) whereas the cultures grown until day 45 were washed three times with isotonic sodium chloride (NaCl) solution after aspirating the cell medium. All the cells were then fixed with 4 % formaldehyde (Fisher Scientific 11586711) for 15 minutes at room temperature (RT) and washed three times with DPBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). The fixed cultures were then incubated in blocking buffer: DPBS (without $\text{Mg}^{2+}/\text{Ca}^{2+}$) with 0.1 % (vol/vol) Triton X-100 (Sigma cat # T8787-50 ml) and 5 % (vol/vol) donkey serum (Biowest cat # S2170-100) to avoid unspecific binding of secondary antibodies for 1 hour at 37°C. Subsequently, a primary antibody against TUJ1 (mouse, 1:1000), GABA (rabbit, 1:1000), MAP2 (mouse, 1:1000), NKX2-1 (rabbit, 1:200) were added, and the samples were incubated overnight at 4°C on a shaker. Subsequently, the cells were washed three times with DPBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). Secondary antibodies (1:500) and DAPI (1:1000) were then applied and the cultures were incubated for 2 hours at RT with mild agitation (Kaur et al, 2016; [4]). References for the antibodies used are given in Supplementary Table 2. Cultures were washed three times with DPBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$) and imaged with a Zeiss LSM900 microscope. To obtain a comprehensive view of the various diamond scaffolds treated in different ways, imaging was performed from both below and above after extraction and flipping it onto a clean glass coverslip.

For neurite length measurements, we utilized the Zen Blue length measurements tool. To measure the number of somata adhering to the cantilevers, we employed Stardist 2D using regions of interest (ROIs) of size 130 $\mu\text{m} \times 1000 \mu\text{m}$. For intensity measurements, we used the Fiji ImageJ software to analyze 100 $\mu\text{m} \times 500 \mu\text{m}$ ROIs.

2.10. Scanning electron microscopy

On day 45, the cell cultures were initially rinsed with isotonic 0.9 % NaCl solution to remove any residual debris or contaminants. The cells

were subsequently fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate at room temperature for 2 hours. Subsequently, the samples were washed thrice with 0.1 M sodium cacodylate for 10 minutes each. To further stabilize the samples, they were immersed in 1 % osmium tetroxide (OsO₄) in 0.1 M sodium cacodylate for 2 hours. The cultures were rapidly rinsed thrice with distilled water to remove any excess chemicals. Later, the samples were dehydrated using a series of ethanol solutions, beginning with two washes in 30 % ethanol for 10 minutes each, followed by sequential washes with higher concentrations of ethanol (40 %, 50 %, 60 %, up to 100 %, 10 minutes each). The samples were then dried using a critical point dryer to ensure that the cellular structure was preserved. To enhance the conductivity of the samples, they were coated with 6 nm of gold using sputter-coating. Finally, the samples were imaged using an FEI Quanta 3D FEG SEM to visualize the cellular structure at high resolution.

The area covered by the interneurons was calculated by the plugin Weka Trainable 2D segmentation in Fiji software, creating two classifications, diamond background and diamond covered by interneuron stem cell culture.

2.11. Statistics

All the data were analyzed using GraphPad Prism 9 software. The data were expressed as the mean±SEM, where the mean was depicted by the white dashed line in the violin plots. Note that n represents the number of replicates analyzed per group. A normality test was used to determine whether the data were parametric or non-parametric. For parametric data, an unpaired t-test with Welch correction was applied by presuming unequal population variances or sample sizes. If the data were non-parametric, the Mann–Whitney test was applied. For multiple comparisons, one-way analysis of variance (ANOVA) test with Welch correction was used for parametric data, and Kruskal–Wallis one-way analysis of variance on ranks was used for non-parametric data. A P value < 0.05 was considered to indicate statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001).

3. Results

3.1. Fabrication of the nanopolycrystalline diamond scaffold

To produce ultrathin diamond scaffolds for regenerative medicine, they were fabricated [34,35] by growing 1 µm layer of silicon dioxide (SiO₂) via wet thermal oxidation on both sides of a Si wafer (Fig. 1a). A 100 nm stoichiometric nitride layer was deposited on both sides via low-pressure chemical vapor deposition (LPCVD). This process was used to facilitate favorable conditions for the first round of diamond growth via hot filament chemical vapor deposition (HF-CVD) [36]. The samples used for surface characterization were kept at this stage. In the next step, tantalum and platinum metal wires were patterned using photolithography and metal lift-off (Fig. 1 a-d). The HF-CVD diamond deposition was repeated to completely enclose the metal traces with a total target diamond thickness of 2 µm. These encapsulated metals did not interfere with the experiments, as they were completely encapsulated by the diamond. Plasma-enhanced CVD with a SiO₂ masking layer was used to outline the scaffolds for plasma etching via a high-bias power oxygen plasma. To release the scaffolds, we etched the initial SiO₂ layer and subjected the mixture to thorough-wafer Si etching using a high aspect ratio deep reactive ion etching (DRIE) process. A buffered oxide dip wet etch was used to remove the remaining SiO₂ masking layers and remnants. At this point, the diamond scaffolds rested on thin Si cylinders attached to the Si frame. The initial nitride layer, which remained as a thin film on the diamond scaffolds, was removed by plasma etching. The surface of the scaffolds was characterized by atomic force microscopy.

3.2. Incorporation of functional groups on the scaffold modified the electron affinity of the scaffold

According to previous reports, changing the electron affinity of the scaffold increased protein adsorption and subsequently promoted greater number of anchoring points for the functional groups of cells leading to enhanced cell attachment [37–41]. Hence, to modify the electron affinity of the diamond scaffolds and enable them to attract functional groups, the hydrogen-terminated surface of diamond scaffolds was subsequently treated by irradiating the surface with ultraviolet (UV) photons (Fig. 1d). After UV irradiation, the resulting functional groups, which included ketones, carboxylic acids, and alcohols, were embedded on the scaffold surface (Supplementary Table 1). A previous study showed a 10 % increase in oxygen deposits after 60 minutes of irradiation [42]. Therefore, we concluded that UV irradiation time of 4 hours in atmospheric air at one bar pressure would generate significant O₃ (Fig. 1d, steps 1-3). O₃ generation using UV wavelengths of 100-240 nm has been reported as a method for depositing oxygen groups onto diamond films in several studies [43–46]. This method has been known to be more stable for oxidizing diamond surfaces as it introduces oxygen groups while simultaneously replacing hydrogen atoms [47]. Possible oxidative reactions included reactions with ketones, alcohols, ethers, aldehydes, carboxylic acids, and various carbonyl groups. The high electronegativity of oxygen changed the surface from partially electroneutral to electronegative. The surfaces of the scaffolds that had only hydrogen- (H+D) or oxygen-termination (O+D) were called bare diamond scaffolds. In contrast, some of these H- and O-terminated scaffolds were coated with various layers of proteins to promote significant cell adhesion to the scaffolds and to promote a growth-stimulating microenvironment after CNS injury. Therefore, the scaffolds were made bioactive by a layer-by-layer immobilization approach with several neuronal growth-promoting molecules [48], which included anti-NOGO-A [49–51], laminin [52], GDNF [53], fibroblast growth factor 2 (FGF2) [54], and poly-D-lysine (PDL) [55] (Fig. 1d, steps 4-6).

3.3. Surface characterization of bare and protein-coated scaffolds revealed successful surface oxidation and protein deposition

To confirm oxygen deposition and protein adsorption and to analyze the chemical composition and structural properties of the bare (hydrogen, H+D and oxygen terminated, O+D) and protein-coated (hydrogen-terminated, H+BioD and oxygen-terminated, O+BioD) diamond scaffolds, we utilized X-ray photoelectron spectroscopy (XPS). Fig. 2a, b, top-right spectra indicated a significant increase in oxygen-termination (O) (peak highlighted in green) compared to hydrogen-termination (H) (the cyan peak), suggesting that ozone treatment successfully promoted oxidation, thereby leading to a transition from neutral to negative electron affinity [46]. Additionally, the carbon (C) peaks shifted toward the binding energies of O-containing C groups, providing further support for the oxidation process (Fig. 2b, bottom-right spectra).

XPS spectra for the protein coating revealed an increase in both O and nitrogen (N) termination, along with a corresponding decrease in C content (Fig. 2b, light and dark grey peaks). These data suggested that extracellular matrix proteins were deposited onto the diamond substrate successfully. The results also suggested steady protein adhesion onto the O-terminated diamond surface, potentially due to an increase in surface energy and the presence of O-containing functional groups.

To further validate and characterize the unoxidized and oxidized surfaces of the scaffolds, atomic force microscopy (AFM) was used (Fig. 2c). The surface images of the bare diamonds and root mean square (Rq) values indicated Rq values of 16.5 nm and 14.85 nm for H+D and O+D, respectively (Fig. 2c, two upper images). The lower Rq value of O+D scaffolds likely resulted from oxidative etching induced by ozone treatment. Additionally, we observed a decrease of approximately 5 nm in Rq between the uncoated and protein-coated diamond samples (H+BioD:

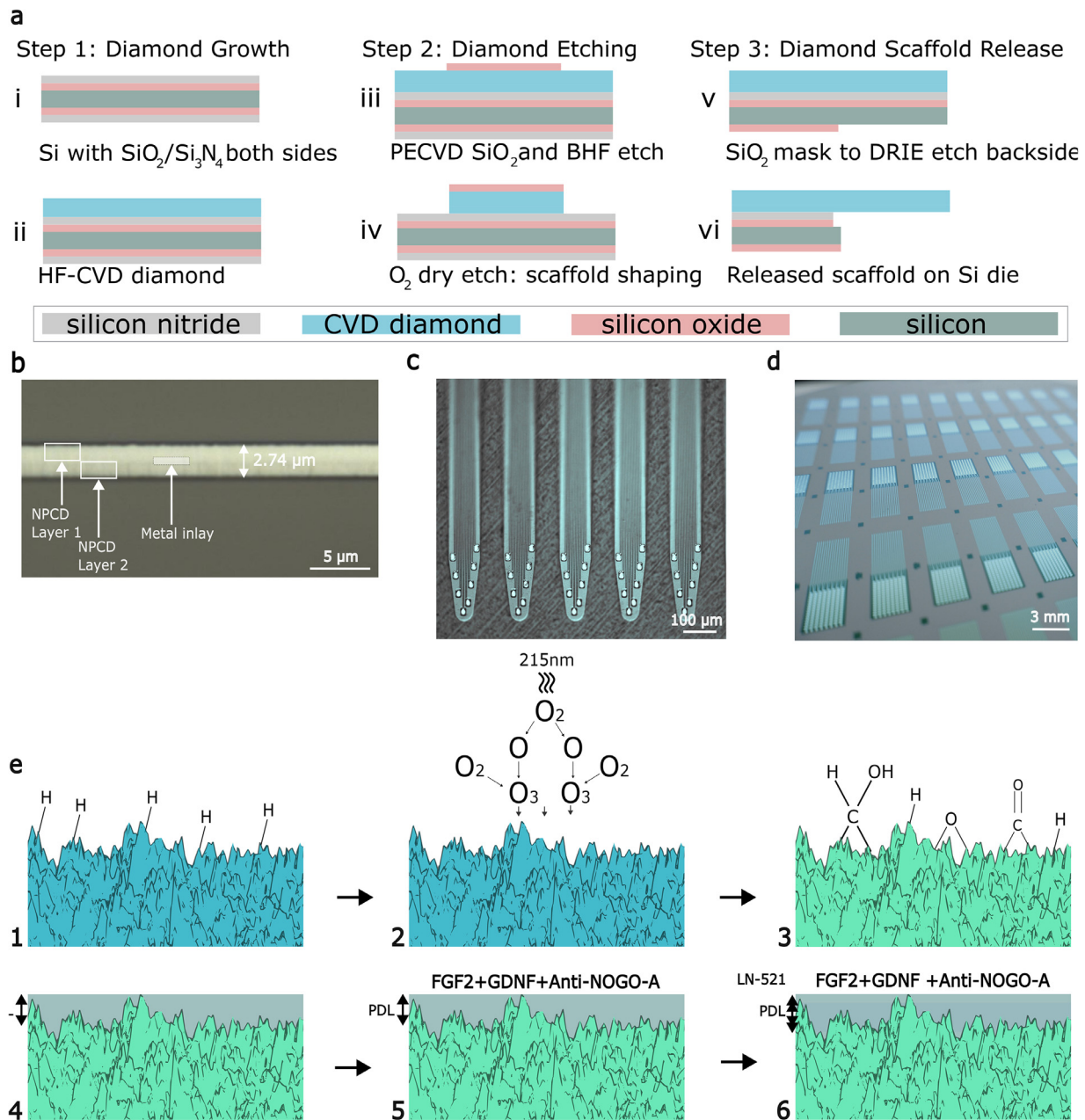


Fig. 1. Fabrication and design of nanopolycrystalline diamond (NPCD) scaffolds with layering of neuronal growth-promoting molecules a) NPCD scaffolds were fabricated in 3 major steps: 1) HF-CVD diamond growth on the wafer substrate (i-ii); 2) scaffolds were shaped using a silicon oxide mask (iii) and oxygen plasma dry etch (iv); and 3) finally, the Si was etched via a backside deep reactive ion etching (DRIE) process (v-vi) to have a free-hanging diamond scaffold resting on a Si die. b) Side view of a single NPCD cantilever, indicating the three layers (the metal inlay enclosed in the NPCD layers). The cantilever thickness was 2.74 μm . c) NPCD scaffolds with 5 cantilevers are shown (optical microscope, 10X magnification). d) Multiple diamond scaffolds on a wafer (dimensions: 5 mm $\times$ 110 μm , 8 nanofibers/cantilever with 16 cantilevers per scaffold). e) Layering of the protein coating where the hydrogen- (H-) terminated surfaces of the diamond scaffolds (1, in cyan) were oxidized (2, in cyan) and the other groups were also expected to be deposited onto the scaffold (3, in green) and here, the scaffold was called the bare scaffold (with either H- or O-termination). Further, the H- and O-terminated scaffolds were layered with poly-D-lysine (PDL, 4), FGF2, GDNF, anti-NGO-A coatings and laminin-521 (LN-521, 5, 6) coatings, and the resulting scaffold was called bioactive (O+BioD and H+BioD).

$R_q = 10.36 \text{ nm}$, O+BioD: $R_q = 9.32 \text{ nm}$), which we attributed to the protein coatings on the diamond surface. Here, note that the measurements were performed on diamond slabs; thus, the measured R_q values were extrapolated to the diamond scaffold.

3.4. Scaffold-cultured interneurons exhibit GABAergic identity and high viability

To reveal MGE interneuron identity, we used a differentiation protocol adapted from Nolbrant et al [33]. Here, we first performed qPCR at

progenitor (16 days) and mature neuronal (42 days) stages (Fig. 3a-b). qRT-PCR results showed high levels of mRNA levels for ventral forebrain progenitor markers *NKX2-1* and *FOXP1* at day 16 whereas more mature interneuron markers such as *ASCL1*, *ARX* and *LHX6* were expressed at lower levels. In contrast, after maturation (at day 42), mRNA levels of *NKX2-1*, *FOXP1* genes were lower whereas mRNA levels of *ASCL1*, *ARX* and *LHX6* genes were higher, highlighting their differentiation into mature GABAergic interneurons. To further validate the identity of the differentiated interneurons, we performed immunocytochemistry (Fig. 3). Day 42 neurons showed clear expression of pan-neuronal marker MAP2

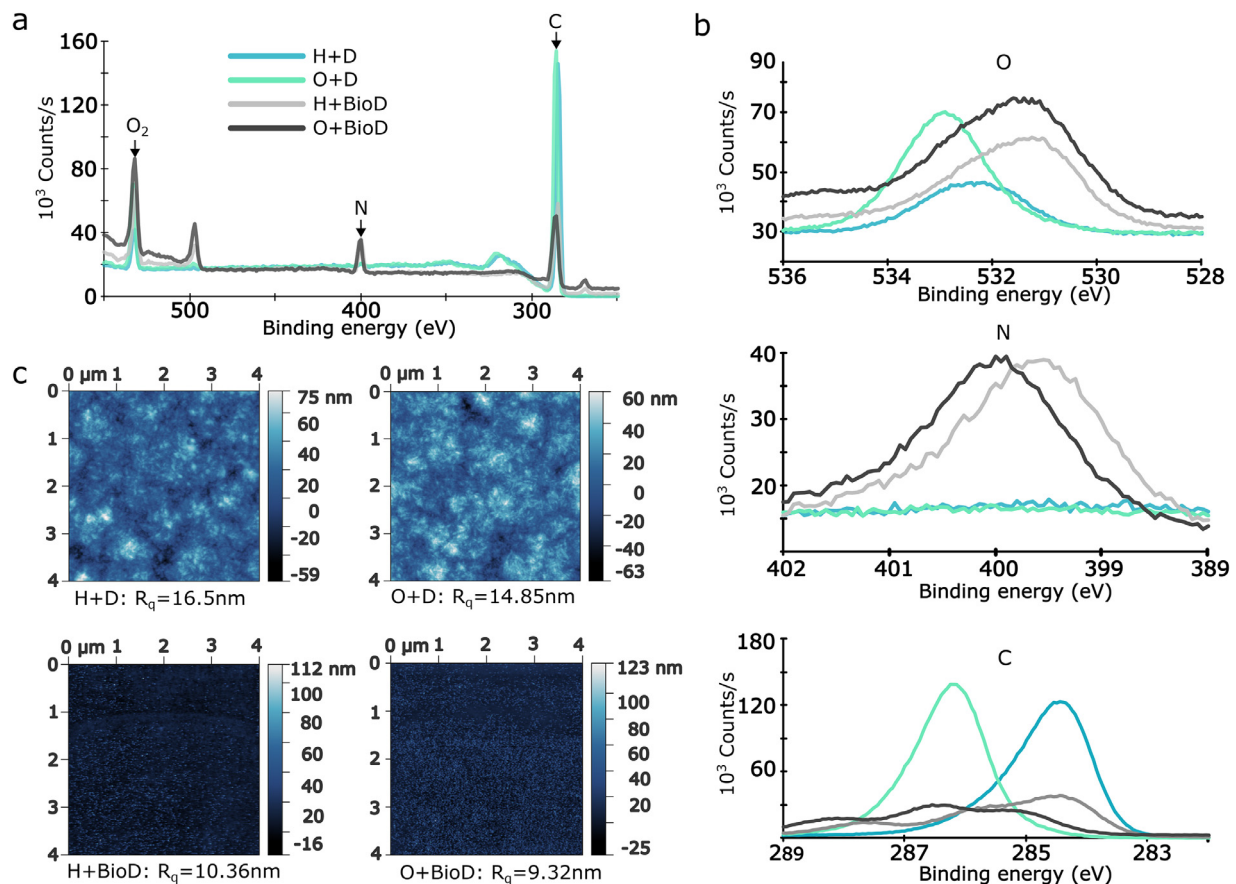


Fig. 2. Evaluation of the hydrogen (H-) and oxygen (O-) terminated surfaces of diamond scaffolds without and with protein coatings. a) XPS demonstrated that higher counts/s of O and nitrogen (N) and lower counts/s of carbon (C) were relevant for surface oxidation and organic matrix deposition (peaks). b) Oxygen peak binding energies (top right plot) showing the highest number of O atoms found on the bioactive diamond surface (O+BioD) and nitrogen peak binding energies (middle right plot) showing N deposition on the surface of bioactive diamonds (H+BioD and O+BioD), whereas carbon peak binding energies (bottom right plot) depicted the maximum C atoms found on the surface of bare diamonds (O+D and H+D). c) AFM images of the H- and O- terminated surfaces of a diamond scaffold without protein coating (two upper images), with heights ranging from -59 nm to 75 nm and from -63 nm to 60 nm and an R_q values of 16.5 nm and 14.85 nm (mean of two examined samples), respectively. The H- and O- terminated surfaces were coated with organic compounds (H+BioD and O+BioD, lower panels), with heights ranging from -16 nm to 112 nm and from -25 nm to 123 nm, where an R_q values of 10.38 nm and 9.32 nm respectively (mean of two examined samples). Small artifacts up to 112 nm and 123 nm were evident due to the coating in H+BioD and O+BioD respectively.

(in green), neurotransmitter GABA (to label GABAergic neurons in red) and 4',6-diamidino-2-phenylindole (DAPI, to label nuclei in blue), further demonstrating interneuron identity.

Finally, to demonstrate whether interneuronal viability was affected when grown together with various scaffolds, we performed a cell viability assay at day 40 and used Hoechst to label live cells and PI staining to label dead cells (Supplementary Fig. 1). The results showed that interneurons grown together with diamond scaffolds in four different conditions (including H+D were 94 %, H+BioD were 82 %, O+D were 94 %, and O+BioD were 90 % viable) were as viable as control cultures (Ctrl, 80 % viable) without any treatment.

3.5. Protein coating facilitates dense interneuronal growth on scaffolds

As an *in vitro* model of axonal regeneration, we applied hESCs to derive MGE interneurons [33] (Fig. 4). hESCs were incubated until the interneurons acquired the neuronal morphology reported to be seen on day 23. As we desired the cultures to acquire neuronal network morphology with longer neurites, we chose to grow the interneurons until day 35 [33]. On day 35, MGE interneurons were treated under various conditions, including 1) control (Ctrl) cultures with no scaffolding treatment, 2) cultures with bare H+D scaffolds, 3) cultures with bare O+D scaffolds, 4) cultures with bioactive, H+BioD, scaffolds, 5) cultures with bioactive, O+BioD, scaffolds, 6) cultures with 3 mm of mechanical insult

created by pulling sharp forceps through the middle of the cultures, 7) cultures with bioactive, H+BioD, scaffolds placed on the interneurons 4 hours after insult, and 8) cultures with bioactive, O+BioD, scaffolds placed on the interneurons 4 hours after insult. Seeded neurons were further cultured on the scaffolds after the treatment until day 45, after which the cultures were fixed with 4 % paraformaldehyde (PFA) on day 45 and further processed for structural analysis.

We used SEM to investigate the structure, density and morphology of MGE interneurons under different conditions. Fig. 5a shows that Ctrl MGE interneurons (without any scaffold) formed a dense distribution of mature neurons where cell clusters were visible (Fig. 5a, image 1-3 in grey). The fourth column of images shows interneuronal density in terms of area (Fig. 5a) where the Ctrl group indicated a high density of neuronal growth. However, bare scaffolds, whether they were treated with H (H+D, Fig. 5b) or with O (O+D, Fig. 5d) exhibited almost negligible attachment to interneurons. In contrast, protein-coated scaffolds (H+BioD and O+BioD) exhibited substantial interneuronal attachment and growth (Fig. 5c and e). Violin plot (Fig. 5f) depicts % area covered by mature interneurons when grown in a dish in Ctrl condition, without and with scaffolds. About 94 % of area covered by Ctrl interneurons showed survival and maturation, whereas only 1.5 % and 1.8 % area was covered by interneurons when grown on bare H+D and O+D scaffolds respectively (Ctrl vs H+D: *** $P < 0.0001$; Ctrl vs O+D: $P < 0.0001$; Welch's t-test). In contrast protein coating of scaffolds highly improved

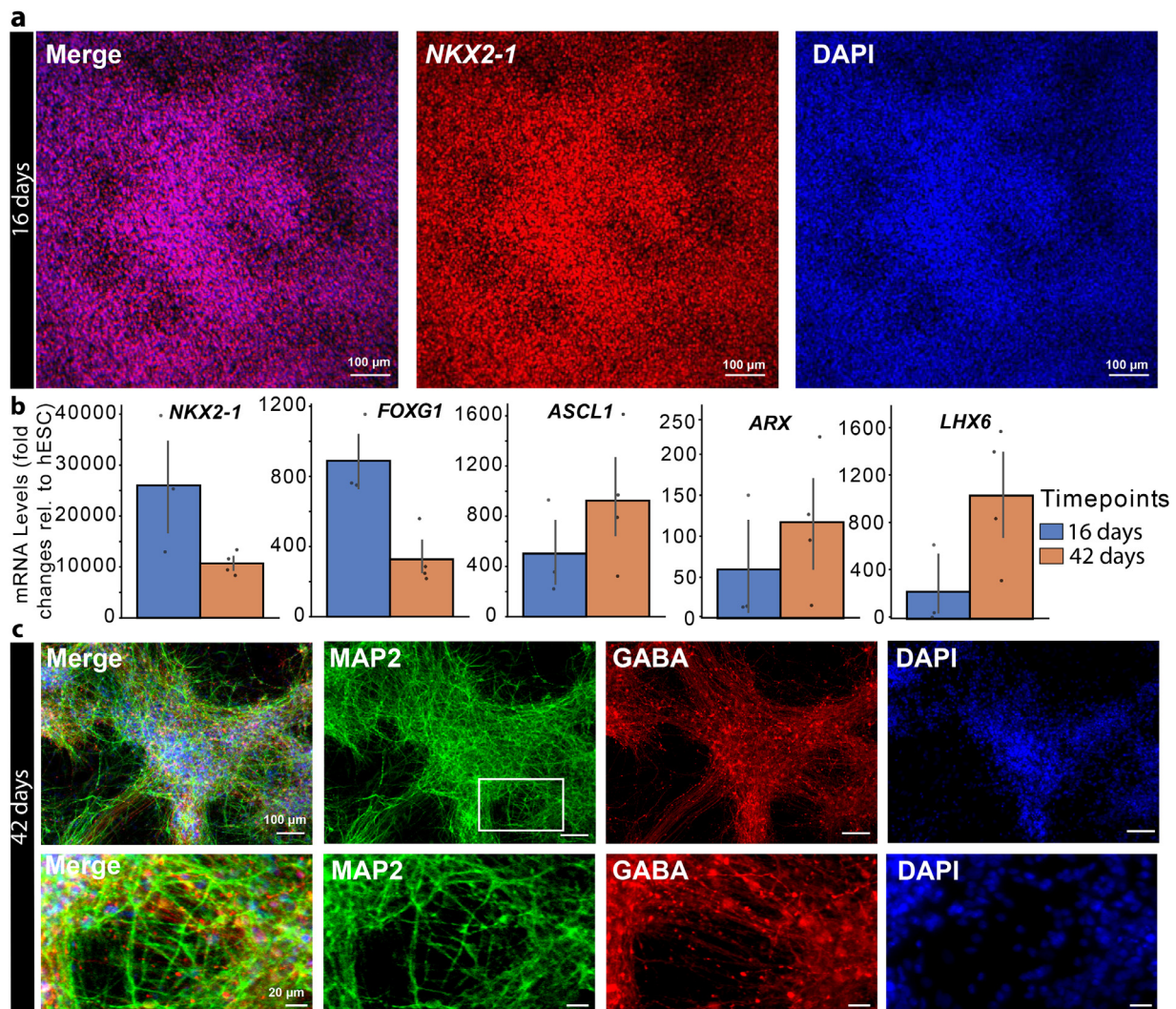


Fig. 3. MGE interneurons were GABAergic. a, b) Example images and mRNA data showing progenitor MGE interneurons at 16 and matured interneurons 42 days where the mRNA expression of *NKX2-1* and *FOXG1* was higher and *ASCL1*, *ARX* and *LHX6* was lower at day 16. However, the expression level of *NKX2-1* (day 16 vs day 42, *t*-test, $P=0.0683$), *FOXG1* (day 16 vs day 42, *t*-test, $P=0.0116$), was lower and *ASCL1* (day 16 vs day 42, *t*-test, $P=0.2995$), *ARX* (day 16 vs day 42, Mann-Whitney test, $P=0.4000$) and *LHX6* (day 16 vs day 42, *t*-test, $P=0.0840$) were higher at day 42 suggesting GABAergic identity (number of samples were 3-4). c) Immunocytochemistry data confirming the neurons (stained by MAP2) were GABAergic by GABA staining and nuclei by DAPI staining. Scale bar = 100 μ m for upper panel. Zoomed-in images of GABA, MAP2 with neurite extension and DAPI staining as shown in lower panel with the scale bar = 20 μ m. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

adherence and survival of interneurons as shown by an increased percentage area to 48.4 % for H+BioD and 76.5 % for O+BioD scaffolds (Ctrl vs H+BioD: $P<0.0001$; Ctrl vs O+BioD: $P=0.0005$). Interneurons covering lowest percentage area when grown on bare scaffolds indicated lack of interneuronal adherence to the scaffolds. In contrast, interneurons covering an increased surface area on the bioactive scaffolds indicated that protein coating facilitated interneuronal adherence, survival and outgrowth on the scaffolds. Additionally, O-terminated bioactive scaffolds showed significantly greater neuronal growth than H-terminated bioactive scaffolds (Fig. 5c, e and f; H+BioD vs O+BioD: *** $P<0.0001$) which indicated that O-termination together with the protein coating provided a favorable microenvironment for interneurons to attach and to encourage growth on the scaffolds.

3.6. O-terminated bioactive scaffolds promoted elongation of neurites with minimal pyknosis

To determine which scaffold offered the most favorable environment for neurite elongation with minimal cell death, we performed immuno-

cytochemistry for TUJ1 to label neuronal processes and DAPI to label nuclei, including also nuclei undergoing pyknosis (i.e., the condensation of nuclear chromatin) (Fig. 6a-c, Supplementary Figure 2a-c showing zoomed-in images). Using brightfield microscopy (BF), neurons were found at the top and bottom surfaces of the scaffolds (Fig. 6a-c, column 1). Widefield fluorescence images of the Ctrl group (with no scaffold) showed short neurites (Fig. 6a), whereas the top and bottom surfaces of H+BioD had long neurites, followed by O+BioD scaffold which had longer neurites (Fig. 6b, c, column 2; Fig. 6d-e; H+BioD vs O+BioD; plot 1 top: $P=0.027$; plot 1 bottom: $P=0.0213$). However, the immunofluorescence intensities of the cultures grown with the H+BioD and O+BioD scaffolds were similar to Ctrl culture on the top surface (Fig. 6d, plot 2), which implied that the density of neurites growing under these 3 conditions was similar even though the length of the neurites varied. In contrast, the bottom surface of the cultures grown with O+BioD scaffold showed enhanced immunofluorescence intensity of TUJ1 (Fig. 6e, plot 2; H+BioD vs O+BioD: $P=0.0376$), indicating a favorable neurite elongation environment provided by O+BioD. TUJ1 length data indicated that O+BioD scaffolds not only provided favorable conditions for the

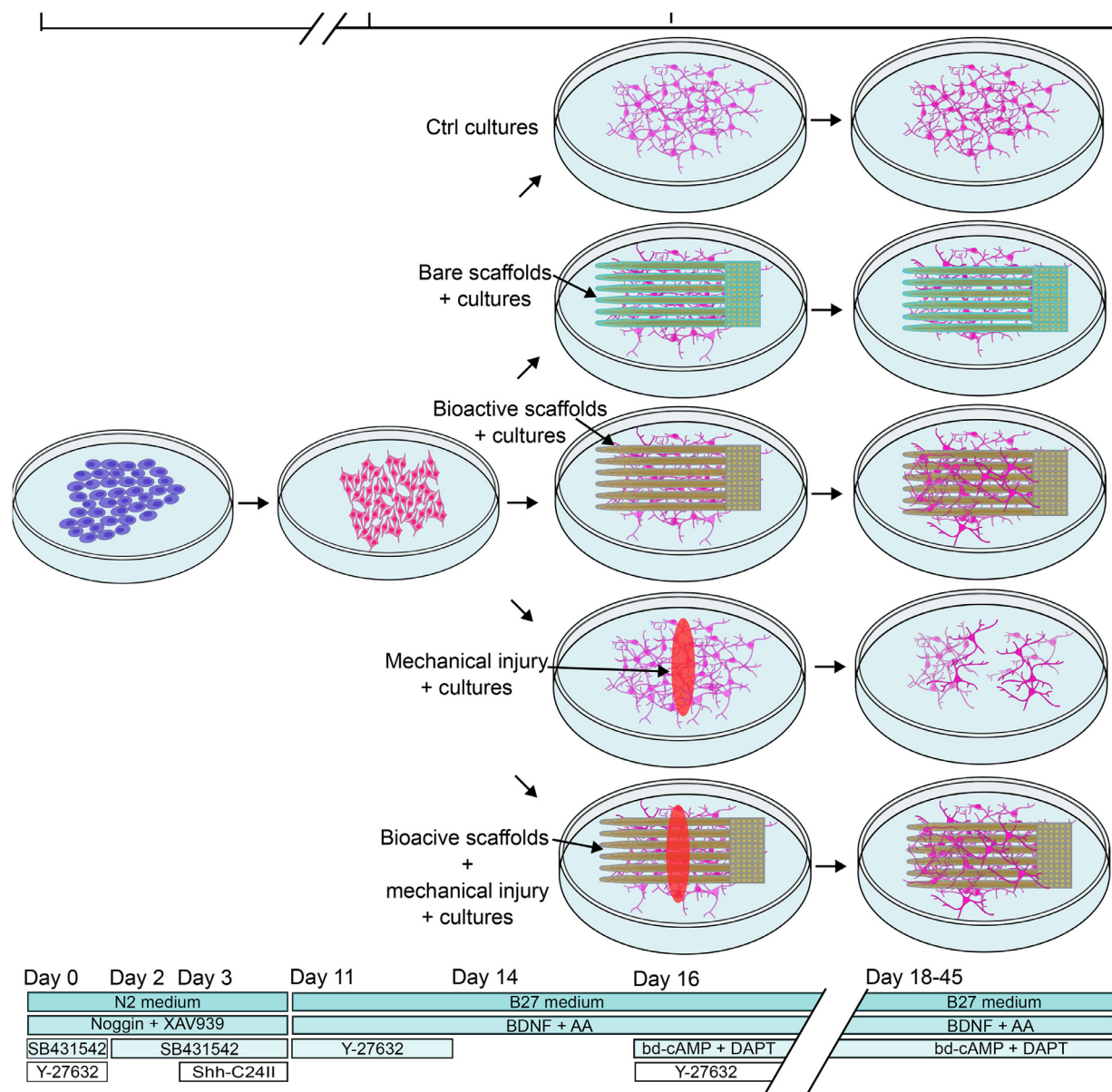


Fig. 4. Stages of the differentiation protocol for hESC-derived MGE interneurons and the placement of diamond scaffolds in culture under various conditions. On day 16, the progenitors were ready for neural induction, following maturation until day 35. a) On day 35, for comparison interneurons were grown undisturbed in a glass Petri dish (Ctrl). b) Bare scaffolds (H+D and O+D) were placed on the interneurons until day 45. c) Bioactive scaffolds (H+BioD and O+BioD) were placed on the interneurons until day 45. d) A cut was created in the middle of the culture to mimic an injury. e) A subset of these cells was supplied with a bioactive scaffold placed on the cells until day 45. f) Scheme of all the media and factors used to grow MGE interneurons starting from day 0 to day 45.

neurons to extend to longer lengths but also demonstrated their usefulness as a novel material with a suitable atmosphere for human neuronal regeneration. Similar TUJ1 intensities on the top surface for H+BioD, O+BioD and Ctrl culture groups implied that a greater density of neurons was growing on the bioactive scaffolds, which also indicated that the bioactive scaffolds were biocompatible. Additionally, higher intensity of TUJ1 on the bottom side of the O+BioD scaffold further demonstrated their higher biocompatibility.

Moreover, we quantified the percentage of pyknotic nuclei, the number of nuclei and their immunofluorescence intensity. O+BioD scaffolds exhibited only 1.5% pyknosis on the top and 7.8% pyknosis on the bottom side (Fig. 6d, e, plot 3 top: Ctrl: 5% pyknosis, H+BioD: 7.6% pyknosis; plot 3 bottom: Ctrl: 5% pyknosis, H+BioD: 11.8% pyknosis) together with a significantly higher number of nuclei on the bottom side (Fig. 6e, plot 4 bottom, H+BioD vs O+BioD: ** $P=0.002$) and a higher immunofluorescence intensity of nuclei on both sides (Fig. 6d-e, H+BioD

vs O+BioD; plot 5 top: $P=0.0017$; plot 5 bottom: $P<0.0001$) suggesting that O+BioD scaffolds provided healthier and suitable conditions for the neurons to adhere and elongate neurites.

3.7. Bioactive scaffolds facilitated directional regeneration of interneurons after mechanical injury

To determine the regeneration capacity of the bioactive diamond scaffolds, we induced mechanical injury in the cultures on day 35 by using a forcep 3 to induce a larger injury size of 5 mm $\times$ 1 mm. The scaffolds were placed perpendicular to the injured area (to bridge the injury) 4 hours after the injury (Fig. 7). 4 hours delay in placing the scaffolds after the injury was chosen based on the following factors: 1) 4 hours time was chosen to mimic cascades of secondary injury, and 2) to mimic the delay that could occur in arriving at the hospital after an accident. After the scaffold was placed on the interneu-

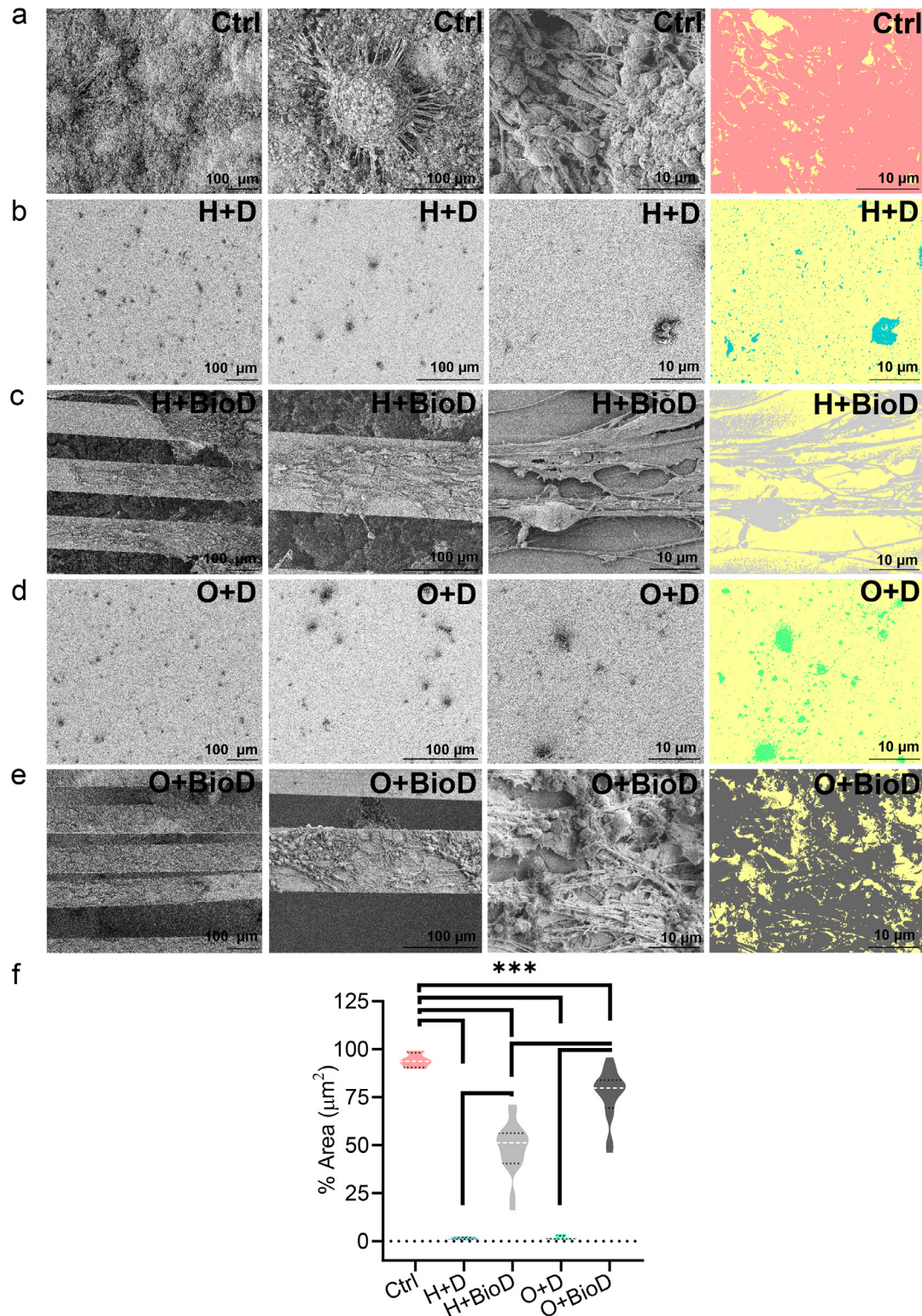


Fig. 5. Structure and morphology of the seeded interneurons on the diamond scaffolds. a) Control interneurons (Ctrl, $n=4$ samples) exhibited a dense distribution of neuronal growth (left to right: SEM at 150X, 1200X, and 8000X magnifications). Right row: Area covered by interneurons with respect to the background area. b and d) Cultures with H- and O-termination and no protein layer (H+D, $n=8$; O+D, $n=7$) indicated a lack of interneuronal adherence. c and e) H+BioD ($n=20$) and O+BioD ($n=15$) scaffolds exhibited significant interneuron attachment and proliferation. f) Violin plot depicting that control (Ctrl) interneurons and interneurons grown with protein-coated scaffolds covering a larger area than the bare scaffolds (Ctrl vs H+D: $***P<0.0001$; Ctrl vs H+BioD: $P<0.0001$; Ctrl vs O: $P<0.0001$; Ctrl vs O+BioD: $P=0.0005$; Welch's t-test, two-tailed). Interestingly, O+BioD showed the highest coverage area with interneurons (H+BioD vs O+BioD: $P<0.0001$, Welch's t-test, two-tailed; $P<0.0001$). ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Scale bars from left to right = 100 μm , 100 μm , 10 μm and 10 μm .

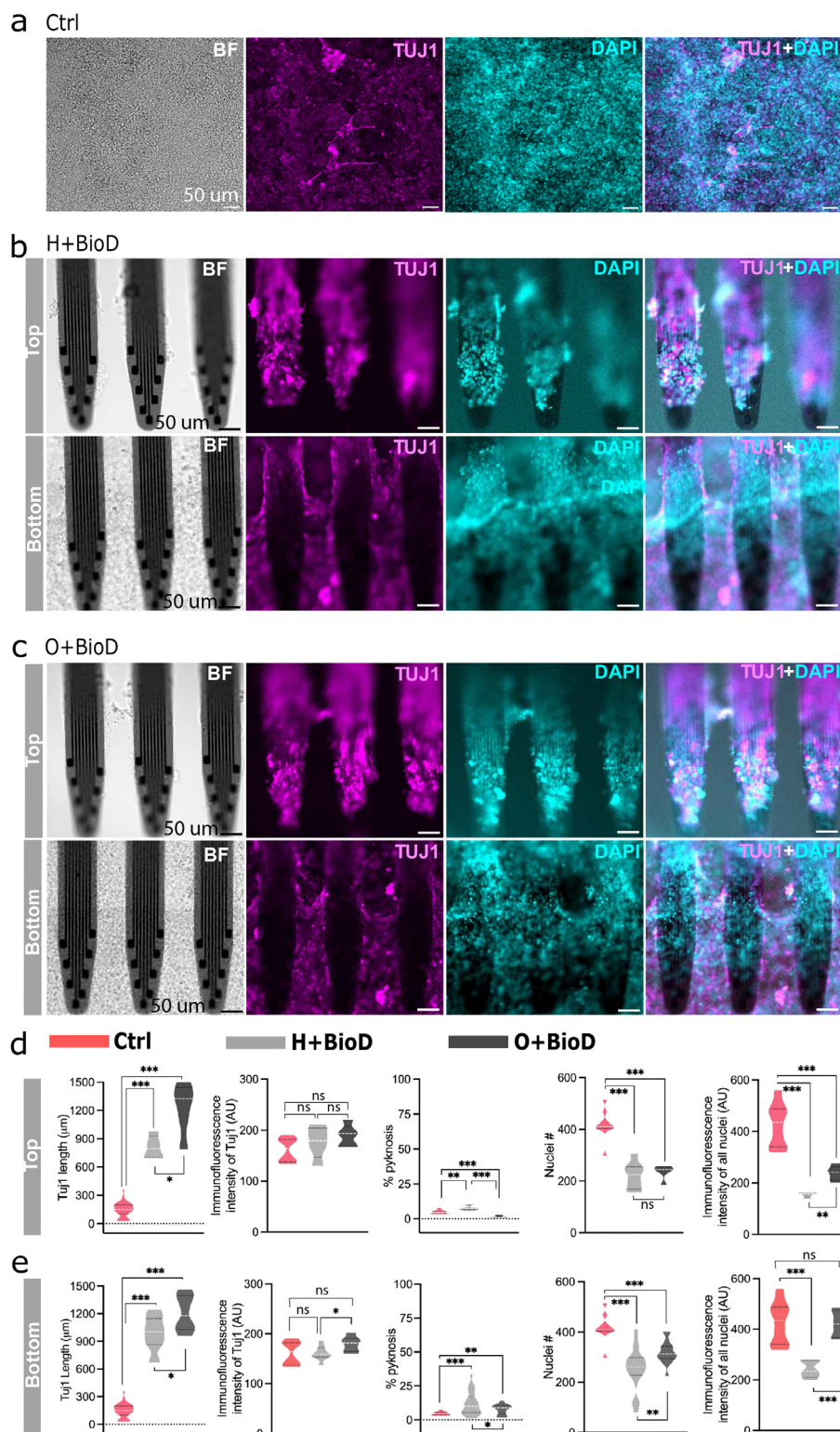


Fig. 6. Attachment and growth of MGE interneurons on diamond scaffolds. a) Widefield images (10x) were acquired from the control interneurons (Ctrl), revealing a uniform distribution of interneurons. b, c) Fluorescence microscopy of H+BioD and O+BioD groups demonstrating successful adhesion, growth, and extension of neurites on the scaffold surfaces (both top and bottom sides). d-e) Quantification of images suggesting that the length of neuronal processes substantially increased, as indicated by staining for tubulin (TUJ1, magenta) on the bottom side of O+BioD. Pyknosis (DAPI staining in cyan) was minimum in the O+BioD scaffold group where the number of nuclei was significantly greater with a higher intensity of neurons, exemplifying greater biocompatibility of O+BioD scaffolds than H+BioD scaffolds. Number of samples analyzed, $n = 10-12$. Scale bar = 50 μm . (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

rons, they were grown for an additional 10 days until the day 45 and fixed with 4% PFA for immunohistochemistry. Injured interneurons grown alone until day 45 displayed very short neuronal processes regrown in a randomized manner according to the TUJ1 biomarker (Fig. 7a and Supplementary Figure 2d-f showing zoomed-in images). However, H+BioD and O+BioD scaffold placement over the injured area showed significantly longer neurites (Fig. 7b-c) regenerating along the scaffold and creating a bridge over the injured area (Fig. 7d-e; top

plot 1, Injury vs Injury+H+BioD: $P=0.0015$, Injury vs Injury+O+BioD: $P<0.0001$; bottom plot 1, Injury vs Injury+H+BioD: $P<0.0001$, Injury vs Injury+O+BioD: $P<0.0001$). Nonetheless, the fluorescence intensity of TUJ1 was greater on the top surface after the O+BioD scaffold was placed at the injury site, which suggested that interneurons were able to migrate effortlessly on the top of the scaffold surface (Fig. 7d-e, top plot 2, Injury vs Injury+O+BioD: $P=0.0284$; Injury+H+BioD vs Injury+O+BioD: $P=0.0072$). Consequently, pyknotic nuclei were esti-

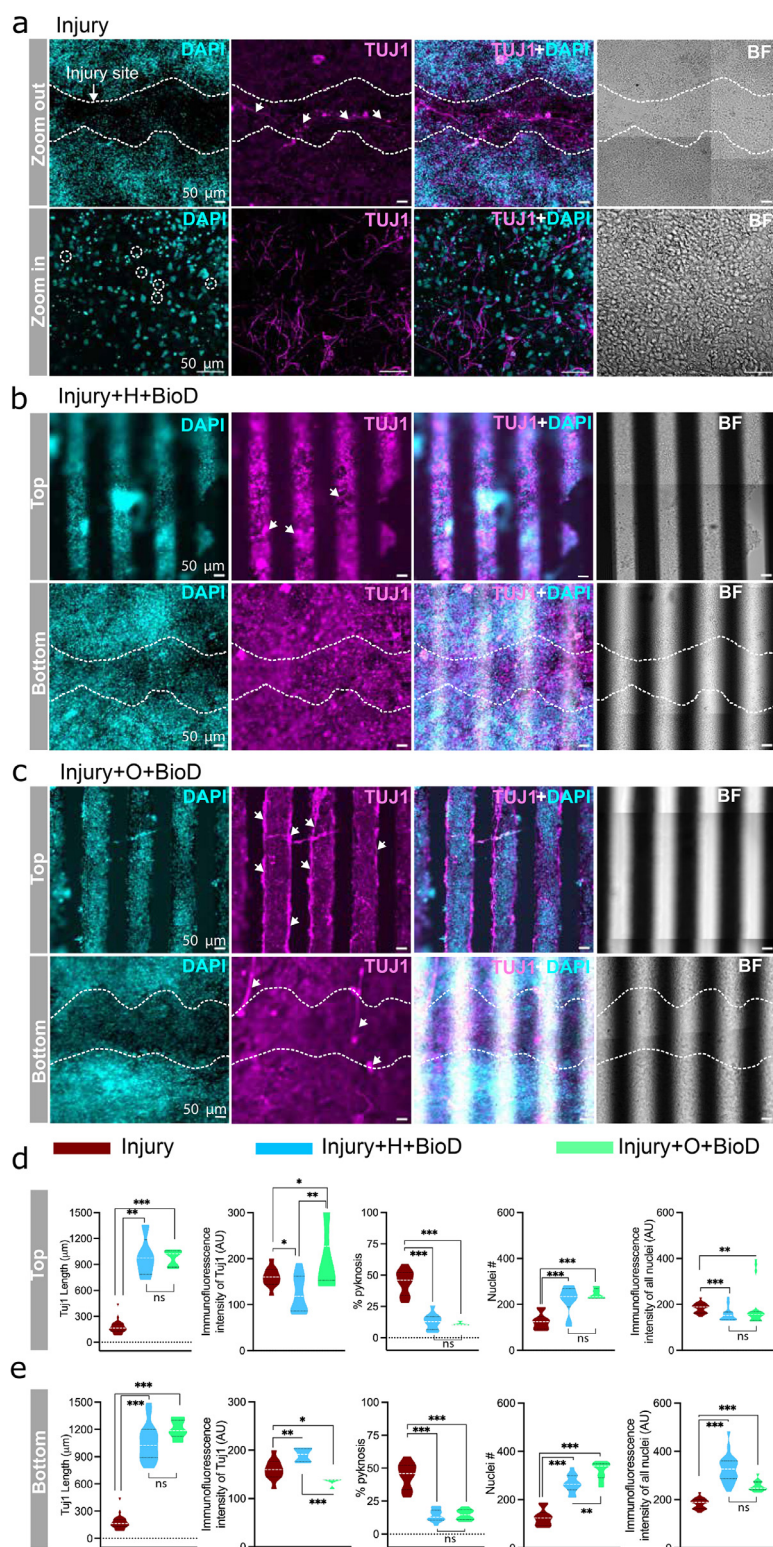


Fig. 7. Following mechanical injury, substantial axonal regeneration was observed after treatment with bioactive scaffolds. a) Widefield (10X, top panel) and confocal (lower panel, 20X) microscopy of nuclei (DAPI in cyan) at the injury site (broken lines), indicating pyknotic nuclei (highlighted in circles) and spontaneous and disorganized cellular growth (TUJ1 in magenta) 10 days after the injury site respectively. b, c) H+BioD and O+BioD scaffolds were placed at the injury site, where the top view showed adhesion and proliferation of nuclei and neurons, and the bottom view showed greater intensity of nuclei and neurite staining (TUJ1) at the injury site (broken lines), which signified neuronal proliferation after the placement of H+BioD and O+BioD at the injury site. d-e) Analysis of the top surface of interneurons suggested that the inclusion of H+BioD and O+BioD surfaces significantly extended neuronal projections; however, the intensity of TUJ1 was greater in case of Injury+O+BioD on the top surface, indicating a greater survival rate of neurons. After incubating the injured cultures with O+BioD scaffolds, percentage of pyknosis was significantly reduced and nuclei number was increased, indicating that O+BioD scaffolds promoted increased recruitment of neurons. In contrast, the total intensity of nuclei was similar for both H+BioD and O+BioD scaffolds. Here, the total fluorescence intensity of the nuclei was measured for both live and pyknotic nuclei. Number of samples analyzed, $n = 10-13$. Scale bar = 50 μm (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

mated, and after the placement of H+BioD and O+BioD, the percentage of pyknotic nuclei was strongly reduced from 45 % to 13 and 11 %, respectively, on the top surface and 14 and 14.5 %, respectively, on the bottom surface. Moreover, there was a significant increase in nuclear number, especially on the bottom surface of the O+BioD scaffold. The increase in total fluorescence intensity of nuclei indicated increased neuronal migration and outgrowth in response to the addition of both H+BioD and O+BioD scaffolds, with the optimal effects of the O+BioD

scaffold after injury (Fig. 7d-e, top plot 4 top, Injury vs Injury+H+BioD: $P=0.0009$, Injury vs Injury+O+BioD: $P<0.0001$; plot 4 bottom, Injury vs Injury+H+BioD: $P<0.0001$, Injury vs Injury+O+BioD: $P<0.0001$, Injury+H+BioD vs Injury+O+BioD: $P=0.0027$; top plot 5, Injury vs Injury+O+BioD: $P=0.007$; bottom plot 5, Injury vs Injury+H+BioD: $P<0.0001$, Injury vs Injury+O+BioD: $P<0.0001$). These data demonstrated that O+BioD scaffolds promoted directional neuronal migration and long-range regeneration across the injury site to reconnect the in-

jured ends, as also shown by TUJ1 staining and indicated by white arrows in Fig. 7b–c.

4. Discussion

The main finding of the current study was the novel demonstration that oxidized diamond scaffolds when coated with neuronal growth-promoting factors (O+BioD) provided a beneficial microenvironment for interneuron stem cells to regenerate and extend their neurites along and across the scaffolds following a mechanical injury. This regeneration and elongation of interneurons over 1 mm in length were observed in 10 days after the placement of O+BioD scaffolds at the injury site. Elongation of interneuron projections was also observed in the control cultures when O+BioD scaffolds were placed. Although H+BioD scaffolds provided a growth-promoting microenvironment to the MGE interneurons, O+BioD substantially boosted neuronal survival and neurite elongation. Using 45-day MGE interneurons, we demonstrated a proof of principle that diamond scaffolds coated with suitable growth factors can serve as a biocompatible platform for the human CNS and enhance neuronal regeneration and survival *in vitro*.

In accordance with the previous reports, the surfaces of bare and bioactive scaffolds were examined using high-resolution XPS and AFM to validate the presence of protein coatings on the scaffolds [56–58]. Hence, using XPS, AFM spectral peaks and surface roughness spectra, we confirmed the presence of H- and O-terminated groups on the bare diamond scaffolds and neuronal growth-promoting molecules on the H- and O-terminated diamond scaffolds, respectively (Fig. 1 and 2). H-terminated but not O-terminated nanocrystalline diamond surfaces reportedly enhance neuronal adhesion and proliferation of mesenchymal stem cells [59]. However, it has previously been stated that more than one cell type can grow on a H-terminated diamond surface, whereas only one cell type can grow on an O-terminated diamond surface [60]. In contrast, Lechleitner et al reported that H-terminated diamond surfaces were unable to adhere cells, whereas O-terminated surfaces demonstrated enhanced cell attachment [61]. Hence to validate the previous reports and also reveal if the diamond scaffolds treated with various conditions affected the cell viability, we conducted a cell viability assay. This assay demonstrated that the cultures were viable in the presence of scaffolds including bare as well as protein coated scaffolds (Supplementary Figure 1). We also observed a few PI-positive dead cells over the scaffolds. This spatial effect of a few dead cells might be due to the local microarchitecture which could create zones of nutrient limitation and stress. This effect has been previously shown in silk scaffolds [62]. However, note that the cell viability in the present study was 80 % in control condition without scaffolds, and introduction of scaffolds has only increased the viability in comparison to control cultures. This increased viability demonstrated that adding scaffolds has supported cell adherence to the scaffold and reduced shear stress as also reported by Guber et al [63]. Furthermore, we identified these MGE interneurons as GABAergic which mediate inhibitory neurotransmission (Fig. 3, [64]).

Structural analysis of interneurons grown on diamond scaffolds by SEM indicated that only bioactive diamond scaffolds (terminated with H or O) promoted neuronal adhesion, recruitment, and outgrowth due to surface coating with proteins [65,66]; however, bare diamond scaffolds terminated with either H or O were unable to support adhesion and outgrowth of interneurons (Fig. 4). Moreover, after the protein-coated diamond scaffolds were placed, the interneurons exhibited a mature neuronal network morphology (by SEM) with cell bodies and extended neuronal projections, such as dendrites and axons, which were elongated to a significant extent. In addition, we observed an increased density of neurites in bioactive O-terminated scaffolds, implying that O-termination accommodated enhanced neuronal connectivity and new circuit formation. In neural stem cells, the growth of neurospheres has been characterized as neuron adherence after the protein adsorption on the ultrananocrystalline diamond [58], which was observed together with neuronal cell bodies and elongated neurites on the bioactive scaffolds (Fig. 5). Furthermore, bioactive scaffolds provided a biocompatible platform for the interneurons to adhere, recruit and migrate.

Interneurons cultured for 45 days exhibited neurite elongation with minimal pyknosis on the bioactive scaffolds and increased neurite length on the O-terminated bioactive scaffolds (Fig. 6, Supplementary Figure 2a–c). Although H-terminated bioactive scaffolds also increased cell survival, H-termination lacked stability due to the risk of coating with groups from the atmospheric air [67,68]. This lack of stability may have induced some variability in the data, and therefore, coating should be performed immediately before placing the scaffolds in the neurons in both *in vitro* and *in vivo* conditions. We suspected that O-termination, if performed hours before using the scaffolds in the experimental settings, could also produce experimental variability. Therefore, any group termination should be achieved immediately prior to the experiment.

Further, neuron-specific beta-tubulin (labelled with TUJ1 antibody, Fig. 6d, e), which marks the period of neurogenesis [69], was highly expressed in neurites elongating on O-terminated than the H-terminated bioactive scaffolds whereas, a minimal neurite length was observed in control cultures (without scaffolds). However, high fluorescence intensity of TUJ1 in control cultures and cultures with H-terminated and O-terminated bioactive scaffolds indicated increased neuronal survival and migration under all three conditions. Nonetheless, an increased expression of TUJ1 on O-terminated bioactive scaffolds (Fig. 6e) could also indicate that the cells may have undergone mitosis and that O-terminated bioactive scaffolds may have promoted neurogenesis and mitosis. O-terminated bioactive scaffolds diminished pyknosis and favored substantially greater cell survival than H-terminated bioactive scaffolds. These observations might be associated with the dense network of neurites present in O-terminated bioactive scaffolds (Fig. 5f), which may lead to increased cell survival.

We further demonstrated that very short neurites of approximately 150–200 microns sprouted in a randomized manner at the injured area (after the introduction of mechanical injury), as shown by TUJ1 biomarker and indicated by white arrows in Fig. 7a. Interestingly, placement of bioactive scaffolds at the site of injury promoted an aligned and directional regeneration of substantially longer neuronal processes extended up to 1.2 mm. The elongated neurites followed the scaffold cantilevers and progressed onto the scaffold, thereby allowing the injured areas of the cultures to cross in a directional manner, as shown by the white arrows in Fig. 7b and c and Supplementary Figure 2d–f. A significant extension of neuronal projections was observed which became even more pronounced after the introduction of the O+BioD scaffold at the injury site, as shown by immunofluorescence intensity measurements of TUJ1 (Fig. 7d, e). Pyknosis was substantially reduced with increased neuronal cell survival after O+BioD scaffold was implanted at the injured cultures. To summarize, bioactive diamond scaffolds particularly O+BioD, followed by H+BioD provided a promising microenvironment for hESC-derived MGE interneurons to attach, migrate, and extend neurites. *In vivo* studies are necessary to validate the findings of the present study in the mammalian CNS system in the future.

5. Conclusion

This study demonstrated a substantial biocompatibility, bioinert and regenerative potential of bioactive diamond scaffolds when placed in hESC cultures. Here the key findings of the study were that: 1) hydrogen-terminated bioactive diamond scaffolds promoted adhesion and survival of hESC-derived interneurons. 2) Although oxygen-terminated bioactive scaffolds boosted substantially greater density of neurons than the hydrogen termination. 3) Interestingly, hydrogen-terminated bioactive scaffolds regenerated across the injury site by regrowing axons. 4) However, oxygen-terminated bioactive scaffolds promoted enhanced and organized regeneration by regrowing significantly longer neurites. This was resulted in bridging the injury gap in comparison to hydrogen-terminated and cultures without scaffolds where neurite regeneration was limited and disorganized respectively. 5) In contrast,

bare hydrogen- or oxygen-terminated scaffolds were unable to support neuronal adhesion of hESCs. In summary, oxygen-terminated bioactive scaffolds provided the most optimal and favorable conditions to regenerate the damaged neurons. Moreover, the results of this study confirmed that these bioactive diamond scaffolds have a translational potential in the future. Hence, preclinical studies are required to reveal the regenerative capacity of the bioactive diamond scaffolds.

Declaration of competing interest

The authors declare no competing interest.

CRediT authorship contribution statement

R J F Sørensen: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nicolas Bertram:** Investigation, Methodology, Writing – review & editing. **Ugne Dubonyte:** Investigation, Methodology, Writing – review & editing. **Bob A Hersbach:** Formal analysis, Investigation, Methodology, Software, Writing – review & editing. **Alison Salvador:** Formal analysis, Investigation, Methodology, Software, Writing – review & editing. **Anpan Han:** Resources, Writing – review & editing. **Agnete Kirkeby:** Funding acquisition, Resources, Writing – review & editing. **Rune W Berg:** Funding acquisition, Resources, Writing – review & editing. **Jaspreet Kaur:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.engreg.2025.07.001](https://doi.org/10.1016/j.engreg.2025.07.001).

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