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Graphene oxide and electroactive reduced graphene oxide-based composite fibrous scaffolds for engineering excitable nerve tissue

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Abstract

This study systematically investigates the role of graphene oxide (GO) and reduced GO/silk-based composite micro/nano-fibrous scaffolds in regulating neuronal cell behavior in vitro, given the limited comparative studies on the effects of graphene family materials on nerve regeneration. Fibrous scaffolds can mimic the architecture of the native extracellular matrix and are potential candidates for tissue engineering peripheral nerves. Silk/GO micro/nano-fibrous scaffolds were electrospun with GO loadings 1% to 10% wt., and optionally post-reduced in situ to explore a family of electrically conductive non-woven silk/rGO scaffolds. Conductivities up to $4 \times 10^{-5} \text{ S cm}^{-1}$ were recorded in the dry state, which increased up to $3 \times 10^{-4} \text{ S cm}^{-1}$ after hydration. Neuronoma NG108-15 cells adhered and were viable on all substrates. Enhanced metabolic activity and proliferation were observed on the GO-containing scaffolds, and these cell responses were further promoted for electroactive silk/rGO. Neurite extensions up to 100 μm were achieved by day 5, with maximum outgrowth up to $\sim 250 \mu\text{m}$ on some of the conductive substrates. These electroactive composite fibrous scaffolds exhibit potential to

enhance the neuronal cell response and could be versatile supportive substrates for neural tissue engineering applications.

Keywords: Neuronal scaffold; silk fibroin; reduced graphene oxide; graphene oxide; electrospinning; neuronal cells

1. Introduction

Nerve tissue is electrically sensitive, and the inhibition of electrical signaling can impede normal tissue function. As electrical integrity is essential for this native tissue, one of the main strategies to enhance regeneration is through electrical stimulation [1–3]. Consequently, extensive effort is invested in improving conduction to provide direct electrical activation to enhance cell and tissue function. A particularly interesting option to improve the outcome of nerve repair is the introduction of endogenous electrical regimes within the conduit lumen. Conductivity can be enhanced by facilitating ionic, protonic or electronic transportation depending on the charged carrier [4–6]. Usually, the introduction of electroconductive moieties in tissue constructs is challenging due to aggregation, limited biocompatibility and poor dispersibility [7,8]. Several conjugated polymers (CPs) based on polypyrrole (PPy), polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT) have been previously investigated to provide scaffolds for nerve regeneration with certain levels of conductivity [9]. However, some CPs such as PPy are difficult to further process once synthesized, they are mechanically rigid and brittle, and insoluble after synthesis [10]. Their use in biological applications, as in PANI, is also limited by their low processibility, lack of flexibility and non-biodegradability [10]. PEDOT, on the other hand, is easily dispersed in aqueous solutions and amenable to solution processing, yet it still shows brittle behavior and tendency to crack, attributed to its tight coil-like conformation in the polymer backbone [11]. In contrast, carbon-

based materials (i.e. graphene family nanoplatelets and carbon nanotubes) have received great attention in recent years due to their high versatility and functionality [12].

Graphene is a strong candidate for developing scaffolds due to its electrical conductivity [13], mechanical strength [14], topographical features and high surface area [15]. Graphene oxide (GO) is more readily dispersible thanks to oxygen-containing functional groups on its surface, and stands out for its easy processability, high affinity to specific biomolecules [16], and appropriate cell-interface interactions [17]. GO-based substrates can support adhesion, proliferation and differentiation of neurons [18–21] without the use of chemical inducers. Schwann cell secretion of neurotrophic factors has also been promoted on GO substrates [22], along with modulation of stem cell differentiation towards the neural lineage [23–25]. Graphene has demonstrated as well potential to improve neuronal cell responses, including cell attachment, proliferation and stem cell differentiation for neural tissue engineering applications [26,27]. In particular, graphene has been shown to promote neuro-specific gene expression, and neurite outgrowth and sprouting in several neuronal-like cells [28–30]. Differentiation of neuronal stem cells into neurons, rather than glia, has also been accelerated on graphene substrates [31]. Schwann cell behavior has also been regulated on graphene-containing substrates by promoting migration, proliferation and myelination [32]. However, the underlying molecular mechanisms behind have remained largely unanswered. Only recently, a study showed that graphene could modulate the active and passive bioelectric properties of the cellular membrane [33].

Most research using graphene family materials are reported on traditional 2D coated surfaces, making them difficult to be developed into suitable 3D assemblies for tissue engineering. Therefore, despite the great potential of the various forms of graphene in neural applications, their use as implantable scaffolds is limited, decreasing their suitability for their clinical

translation and applicability in more biological contexts. Instead, biohybrids manufactured from the combination of native polypeptides found in nature, along with graphene family materials, can offer a multifunctional platform in tissue engineering to develop suitable fibrous scaffolds. Of the many naturally occurring proteins, silks have been extensively investigated in nerve tissue repair and regeneration [34]. In particular, silk fibroin (SF) obtained from *Bombyx mori* silkworms is widely available as a raw material and easily processable. Silk fibroin has demonstrated great potential due to its physicochemical, mechanical and biological properties: these include aqueous preparation, superior mechanical properties, suitable elasticity, adequate angiogenicity and controlled biodegradability [34,35]. Immobilizing graphene on silk via electrostatic adhesion is common, but thin coatings tend to lack the interfacial adhesion required in between the surfaces since graphene lacks polar groups to induce strong interactions [36]. A way around has been to coat silk fibers with GO instead and reduce it afterwards [37–39], with the interfacial strength arising from the polar groups of GO and the amide groups of silk. However, a thorough post-reduction of the GO coating can weaken the interfacial strength with silk and its conductive stability. More recently, graphene flakes were entrapped in between the silk fibers prior to fiber contraction induced by ethanol immersion [36], but the fiber morphology and underlying topography tend to be lost. In a more recent study, silk fibers were coated on reduced GO (rGO) gels into heterostructured composites towards peripheral nerve repair [40]. However, scale-up and shaping into future nerve conduits may be challenging. Composite blends prior to fiber formation might be a more direct approach.

This study explores composite micro/nano- fibrous scaffolds based on SF and GO or reduced GO (rGO). Decoupling the role of conductivity on cell behavior is challenging, since manufacturing scaffolds with similar physico-mechanical properties but different levels of

conductivity is difficult. Hybrid composites were fabricated here by dispersing GO into an SF solution at increasing controlled loadings (1%, 5% and 10% wt.) prior to electrospinning and in situ post-reduction to give electroactive SF/rGO. Micro/nanofibers with a multiscale structure similar to that of the native extracellular matrix have been widely investigated in nerve tissue engineering due to their suitability for neuronal cell growth. We hypothesize that the presence of (r)GO (i.e. GO or rGO) in the system positively influences the neuronal cell response, and that this can be further modulated by the electroactivity attributed to rGO. The biological performance of the scaffolds was assessed with analog neuronal NG108-15 cells over their growth and differentiation phase. To the best of the author's knowledge, this is the first comparative study of silk-based composite micro/nano- fibrous scaffolds with GO and rGO for neural tissue engineering applications.

2. Materials and Methods

2.1 Preparation of silk fibroin

Extraction and purification of silk fibroin from *Bombyx mori* silkworm cocoons was carried out as previously described [41], with some modifications (**Scheme 1A**). Briefly, cocoons (Wildfibers, UK) were dewormed, sliced into small pieces, and degummed in a boiling aqueous solution of 0.02 M sodium carbonate (Na_2CO_3) (Sigma-Aldrich, UK) for 60 min. Degummed silk fibroin fibers were thoroughly washed in deionized water (DI water) and air-dried at room temperature overnight. The fibers were then dissolved in 7.9 M aqueous lithium bromide (LiBr) (Sigma-Aldrich, UK) to form a 16% w v⁻¹ solution, under gentle continuous stirring at 60°C for 4 h. The resulting solution was centrifuged (10,000 ×g, 20 min) and dialyzed (10k MWCO SnakeSkin) (Thermo Fisher Scientific, UK) against 5L of DI water at 4°C for 2-3 days, with regular water changes. The concentration post-dialysis was ~5-6% w v⁻¹. The regenerated silk fibroin (SF) solution was centrifuged again, cast onto polystyrene dishes (2 mL of SF solution

per dish) (Sigma-Aldrich, UK) and dried at 60°C for 2 h in a forced air-circulation oven (Mettler Universal, Germany). The resulting films were subsequently peeled off and kept in sealed vials as stock material.

2.2 Preparation of silk fibroin/graphene oxide solutions

Graphene oxide (GO) flakes (2-DTech, UK) (with lateral dimension and number of layers quoted by the manufacturer as <4 μm lateral width and <2 layers) were used as filler. The GO powder was dispersed in $\geq 98\%$ 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, UK) (**Scheme 1B1**) and sonicated at 80 Hz for 2 h using a temperature controlled (RT) ultrasonic bath (Elmasonic P60H, Germany). Following sonication, the lateral width of GO flakes was determined as described in the Supplementary Information (S.I). SF films were then dissolved in the GO dispersion by mixing for up to 3 days at 250 rpm in an oil bath heated at 50°C. Solutions of 10% w v⁻¹ SF/HFIP were obtained at increasing GO loading: control 0, 1, 5, and 10% wt.

A Preparation of stock SF films

B Fiber spinning and in situ reduction

Scheme 1 (A) Schematic of the extraction and synthesis of silk fibroin. (B) Manufacturing of silk-based scaffolds: (B1) Preparation of silk-based dopes for spinning and (B2) schematic of the electrospinning of SF/GO solutions and in situ post-reduction into SF/rGO.

2.3 Fabrication of graphene oxide/silk fibroin fibrous composites and post-reduction treatment

Fibrous scaffolds of SF/GO blends were fabricated by electrospinning, giving SF/1G, SF/5G and SF/10G substrates. Briefly, the spinning solution was transferred into a disposable 6 mL Normject™ syringe (Thermo Fisher Scientific, SG) capped with a 19G blunt metallic needle (0.686 mm inner diameter) (Terumo, SG), fixed in a SP100iZ single-syringe infusion pump (World Precision Instruments, SG). A voltage of 15 kV was generated by a high-voltage DC power supply applied to the needle. A 15 cm × 15 cm static ground collector covered in aluminum foil was placed at a distance of 10 cm from the tip of the needle, and the solution was dispensed at a constant rate of 0.8 mL h⁻¹. All experiments were performed at room

temperature (~22°C) with relative humidity 25% controlled by a dehumidifier device (EuropAce, SG) inside a closed chamber.

The substrates were annealed by immersion in a bath of 80% v v⁻¹ ethanol for 20 min to induce β -sheet conformational transition, thoroughly rinsed in DI water, and dried for 24 h sandwiched between filter paper (Whatman[®]) (Sigma-Aldrich, UK) to prevent curling or folding. The SF/GO scaffolds were post-reduced in situ (**Scheme 1B2**) by treating them with a 1% w/v solution of L-ascorbic acid (Sigma-Aldrich, UK) at 95°C for 1 h [38,42], giving SF/1R, SF/5R and SF/10R substrates. The scaffolds were subsequently washed thoroughly with DI water afterwards. Unmodified SF scaffolds were also treated under similar conditions to use as control.

2.4 Characterization of the fibrous composites

2.4.1 Morphology and surface topography

Fiber morphology and surface topography of different batches of the scaffolds were examined via field emission scanning electron microscopy (FESEM) (JEOL JSM6700F, SG) at 5 kV with a working distance of ~8 mm. Fiber diameters were determined manually with Fiji software by measuring a minimum of 100 fibers.

2.4.2 Estimation of porosity and water sorption

Water sorption capacity of the scaffolds (n=3 per type) was investigated by weight differences between the hydrated and the dry state (refer to details in the SI). The overall percentage porosity of the scaffolds (n=5 per type) was determined through gravimetric analysis (refer to details in the SI). The gravimetric estimate of the percentage porosity of the scaffolds was then used to model the pore size through statistical considerations derived by Eichhorn and Sampson [43] (refer to details in the SI).

2.4.3 Chemical bond analysis

The conformational changes to the secondary structure of silk were evaluated within the spectrum of the amide I region ranging from 1700 to 1600 cm^{-1} , by means of Fourier self-deconvolution (FSD) as detailed in the SI. The spectrum for each scaffold (n=2 per type) was taken with a Fourier transform infrared (FTIR) spectrometer (PerkinElmer 2000) equipped with a zinc selenide (ZnSe) crystal on attenuated total reflectance (ATR) mode.

2.4.4 Electroconductive properties

An automated electrical conductivity and resistivity system (A4P-200 MicroXACT, US) with a Jandel 4-point probe head (Tungsten Carbide tips, 40 μm tip radius and 1 mm tip spacing) (Fig. S1) was used to determine the conductivity [38,44,45] at different positions across the surface of the scaffolds (n=4 per type). Measurements were conducted at room temperature ($\sim 20^\circ\text{C}$) in the dry state, in the hydrated state, and in the dry state after one-day hydration, as described in the SI.

2.4.5 Raman spectroscopy

The presence of GO and rGO within the scaffolds (n=2 per type) was evaluated using a confocal LabRAM HR Evolution Raman microscope (HORIBA Scientific, SG), by acquiring the Raman spectra at different positions along the surface of the sample (refer to the SI for details).

2.4.6 Profilometric surface analysis

Differences to the surface topography of the scaffolds (n=2 per type) were analyzed at room temperature on a Countour GT-K1 3D optical profilometer (Veeco, USA) with light interferometer (refer to the SI for details) [46]. Analysis of the surface roughness was carried out using Vision64 MapTM (Bruker, USA) software. The arithmetical mean surface height (S_a) and root mean square surface height (S_q) parameters of the 3D profile ordinates were analyzed for each image taken, and the average values for each sample type determined.

2.4.7 Protein adsorption

The capacity of the scaffold (n=3 per type) to adsorb protein was evaluated using Engelbreth-Holm-Swarm murine laminin (Sigma-Aldrich, UK) and the NanoOrange[®] protein quantification kit (Thermo Fisher Scientific, UK), based on the amount of remaining protein in solution after adsorption (refer to the SI for details). Fluorescence intensity was read (ex./em. 485/590 nm) with a FLUOstar Optima microplate reader (BMG Labtech).

2.5 In vitro study

2.5.1 Cell culture of neuronoma NG108-15

In vitro cell culture experiments were conducted using a NG108-15 neuroblastoma × glioma rat/mouse hybrid neuronal-like cell line (ATCC, UK). Cells were subcultured in CellStar[®] tissue culture polystyrene (TCP) treated T25-75 flasks (Greiner Bio-One, UK). Cells were grown with high glucose (4.5 mg mL⁻¹) Dulbecco's modified Eagles medium (DMEM-HG) containing L-glutamine and without sodium pyruvate (Sigma-Aldrich, UK), 10% v v⁻¹ fetal bovine serum (FBS) (Sigma-Aldrich, UK), 1% v v⁻¹ 100× penicillin-streptomycin (Pen/Strep) solution (10,000 IU Pen and 10,000 µg mL⁻¹ Strep) (Sigma-Aldrich, UK), and supplemented with a HAT 50× solution (Thermo Fisher Scientific, UK) to a final working concentration of 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine. Cells were cultured in a humidified atmosphere incubator at 37°C (5% CO₂ and 95% air), with half of the medium changed every 2 days. Cells were used between passage P17-30 after they became 70-80% confluent.

For cell culture experiments, scaffolds (13 mm diameter) were clipped to CellCrown[™] polycarbonate inserts (Scaffdex Oy, Finland) for 24-well plates (vol. 1 mL). Mounted annealed scaffolds were maintained in sterile molecular biology grade water for approximately 24 h prior to cell culture. The scaffolds were sterilized with 70% v v⁻¹ ethanol for 30 min, followed by a

30 min wash in sterile PBS, transferred into sterile, low-binding 24-well plates (Corning, UK) and exposed to UV-light for 20 min (254 nm) inside a biosafety cabinet. Glass coverslips (CV) (12 mm diameter, 0.13-0.16 mm thickness) (VWR, UK) were used as positive controls. To improve cell adhesion, scaffolds were preconditioned overnight in supplemented medium to promote protein adsorption and facilitate cell attachment. Prior to cell seeding, scaffolds were air-dried inside a biosafety cabinet for 60 min to ensure that the cell suspension would not spread around. A cell seeding density of 10,000 cells per well within a ~50 μ L droplet was used, and the scaffolds were left to adhere for ~60 min inside an incubator before adding fresh media. Half of the media was changed every 2 days.

2.5.2 Cell survival

Cellular viability of the various cell-laden scaffolds (n=2 per type) was evaluated at days 3, 5 and 7 using a LIVE/DEAD[®] viability/cytotoxicity fluorescence kit (Thermo Fisher Scientific, UK) (refer to the SI for details). 3D imaging was carried out using a TCS SP8 confocal laser scanning microscope (Leica Microsystems Ltd., UK) at an ex./em. of 494/517 nm for live cells and 528/617 nm for dead cells. Z-stacking images were acquired by scanning three different areas throughout the thickness of each scaffold at 5 μ m per Z-step. Cell quantification was carried out with Fiji software. Data is expressed as the percentage of live cells versus non-viable/dead cells.

2.5.3 Metabolic activity and cellular proliferation

The metabolic activity of cell-laden scaffolds (n=4 per type) was monitored on days 3, 5 and 7 using the alamarBlue[™] reduction assay according to the manufacturer's instructions (refer to the SI for details). The fluorescence (ex./em. 544/590 nm) was measured with a FLUOstar Optima microplate reader (BMG Labtech, UK), and recorded as a function of the dsDNA content to give an indication of the metabolic activity relative to the number of cells.

Cellular proliferation of the same samples at similar time points was then measured by the Quant-iT™ PicoGreen® dsDNA assay (Thermo Fisher Scientific, UK) (refer to the SI for details). The fluorescence (ex./em. 485/520 nm) was measured with a FLUOstar Optima microplate reader (BMG Labtech), and compared with a generated lambda dsDNA standard curve obtained within the range of measured concentrations. The cell number was estimated based on the assumption that a single cell contains around 5 pg of DNA.[47]

2.5.4 Neuronal differentiation

To induce neuronal differentiation, the cell culture medium was changed to a differentiation medium one day post-seeding, using serum-free medium. The culture was maintained for 5 days, with half of the medium removed and replaced with fresh medium every 2 days.

2.5.5 Immunocytochemistry (ICC)

Cell-laden scaffolds (n=2 per type) were harvested, gently washed twice with PBS and treated with 10% v v⁻¹ neutral buffered formalin (NBF) (Sigma-Aldrich, UK) (equivalent to about 4% w v⁻¹ paraformaldehyde) overnight at 4°C as fixative solution. Samples were rinsed three times with PBS, permeabilized with 0.1% v v⁻¹ Triton X-100 in PBS for 15 min and further rinsed three times with PBS. Samples were then blocked for non-specific binding with 1% w v⁻¹ bovine serum albumin (BSA) in PBS for 30 min and rinsed three times with PBS.

To assess the differentiation of neuronal cell bodies, cell-laden samples were labeled with polyclonal rabbit anti-mouse/rat β -tubulin III (Abcam, UK) as primary antibody, and preadsorbed polyclonal goat anti-rabbit IgG H&L Alexa Fluor® 488 (Abcam, UK) as secondary antibody. Samples were incubated at a 1:1000 v v⁻¹ dilution in PBS for 2 h at room temperature, respectively; PBS was used to rinse in between steps. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) blue (1 mg mL⁻¹) (Sigma-Aldrich, UK) at a 1:1000 v v⁻¹ dilution in molecular biology grade water for 2 min at room temperature.

Samples were finally rinsed again with PBS three times. The volume of solution was ~200 μ L per sample to ensure they were completely submerged.

Samples were mounted for imaging on Fisherbrand™ glass microscopic slides (76×26 mm, 0.8-1 mm thickness) (Fisher-Scientific, UK) using ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, UK); an appropriate sized glass coverslip (18×18 mm, 0.13-0.17 mm thickness) (VWR, UK) was then placed over the section of interest and cured at room temperature overnight following the manufacturer's instructions. Samples were imaged using a TCS SP8 confocal laser scanning microscope (Leica Microsystems, UK) at an ex./em. of 340/488 nm for DAPI and 495/519 nm for Alexa Fluor® 488. Z-stacking images were acquired by scanning different areas throughout the thickness of the scaffold at 5 μ m/Z-step. Images were visualized using Fiji software.

2.5.6 Neurite outgrowth

Average neurite length, maximum neurite extension and average number of neurites per neuronal cell body for each scaffold type were analyzed from immunofluorescent micrographs. Only extended structures longer than 30 μ m (measured from the middle of the cell body until the end of the projection) were considered as neuronal processes. High-magnification images were sampled from randomly selected fields of view, with a minimum of 10 neurites analyzed. Quantification was carried out using Fiji software.

2.6 Data analysis

Statistical analysis was performed with GraphPad Prism 8 (San Diego, USA), and datasets checked for normality. Normally distributed data is presented as standard deviation (SD, error bars) of the mean values. For parametric data and multiple comparisons, significance was assessed by one-way ANOVA (one independent variable) or two-way ANOVA (two independent variables) with Tukey's post hoc analysis test. Not normally distributed data was

assessed by Kruskal-Wallis with Dunn's post hoc analysis test. A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

Dissolution of silk fibroin is an important step in reprocessing it into fibers, but there are limited suitable solvents [48]. Here, we dispersed GO in HFIP, followed by sonication, silk fibroin addition and dissolution as described in the methodology. The distribution of the lateral width of GO flakes after 2 h sonication is shown in Fig. S2, with an average size of $167 (\pm 54)$ nm. Longer sonication times can undermine the mechanical properties of GO, primarily attributed to a more aggressive fragmentation of the flakes resulting in an increased oxidation degree and the presence of more inter junctions and defects [49–51]. With this in mind, it was important to optimize a well-dispersed blended SF/GO solution, where the GO flakes could be effectively electrospun alongside silk fibroin to form bead-free fibers (Fig. S3).

3.1 Fibrous morphology

The surface topographies and morphologies of the composite silk-based scaffolds are shown in **Fig. 1A**. Unmodified silk scaffolds appeared whitish in color. Composite silk scaffolds were rendered increasing brown with increased GO loadings. After in situ post-reduction into SF/rGO, the samples turned into various shades of grey tending to black. Morphology of the fibers, as observed under FESEM, was smooth and consistent for every group tested, with an average fiber diameter of around 800 nm (**Fig. 1B**). A decrease in the average fiber diameter due to larger fiber elongation during spinning has been reported with the incorporation of graphene and its derivatives [52,53]. While some differences could be observed compared with unmodified silk, fibers of similar diameter size were obtained with addition of GO up to 10% wt. – all blended solutions spun at similar conditions. Fiber diameter was not significantly affected by post-spinning annealing, but moderately increased ($p < 0.01$) after post-reduction

with ascorbic acid. In this respect, Huang et al. investigated the effects of different reducing agents on the properties of wet-spun GO fibers, and reported hydroiodic acid to result in lower fiber diameter, whereas hydrazine treatment caused larger diameters [54].

3.2 Porosity and water sorption

Scaffolds manufactured by electrospinning usually consists of densely packed fibers with limited inter-fiber spacing and pore diameter [46]. However, a certain level of porosity is required in nerve conduits to allow infiltration of blood vessels and regulate nutrient uptake, oxygen diffusion and waste exchange [55]. Previously reported data has shown that the addition of carbon-based nanomaterials can lead to an increase in the pore diameter and overall scaffold porosity [56,57]. Following the determination of the percentage porosity, it was clear that the presence of (r)GO did not result in this case in major changes in the overall porosity (70-80%) or the estimated pore dimensions (~15 μm) (**Fig. 1C-D**), and that these are a good compromise to provide a cell-friendly conduit lumen [55]. Indeed, scaffolds with excessive pore diameters may facilitate infiltration of non-neural cells along the conduit length and hinder neurite outgrowth [55]. In particular, pore diameters <30 μm in the conduit lumen are ideal to inhibit the invasion of fibroblasts and macrophagocytes for the regeneration of peripheral nerve [58].

Fig. 1 Effect of (r)GO content on the scaffold structure. (A) Representative macroscopic views of the visual appearance of the scaffolds prior to and after post-reduction, and representative FESEM micrographs of the scaffolds; scale bars at 4 mm and 5 μm respectively. (B)

Distribution of the fiber diameter size of the scaffolds before and after ethanol treatment (annealing) and with post-reduction (n=100 fibers). (C) Overall porosity of the scaffolds (n=5 per type) and (D) estimated average pore diameter. (E) Water sorption of the scaffolds (n=3 per type) after overnight incubation at physiological conditions. Differences between the experimental groups were analyzed by two-way ANOVA with Tukey's test, or by Kruskal-Wallis with Dunn's test. n.s non-significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Water sorption is an also an important feature that can influence the properties of the substrate prior to and after implantation. For instance, the void space might be reduced, increasing direct contact between the cells and the material [59]. Although silk fibroin presents a polymeric structure with hydrophobic regions, it has large sorption capacity to hold moisture thanks to its alternating hydrophilic regions [60]. Enhanced hydrophilicity can lead to increased cell attachment [61]. While the hydrophilicity/hydrophobicity of graphene and its derivatives has remained under constant debate over the last few years, it has been recently demonstrated that when graphene is placed in a water-like environment, it presents hydrophilic properties close to those of pure water [62]. Here, all substrates showed great water sorption capability; while some changes were observed with the presence of (r)GO in the fibers, these differences were non-significant (**Fig. 2C**).

3.3 Molecular structure of silk and the effect of graphene derivatives

The addition of (r)GO into the silk system has previously been reported to lead to changes in the amount of β -sheet structures after ethanol treatment [63–65]. The chemical structure of SF was analyzed by FTIR-ATR (**Fig. 2A**). There was no obvious difference in the spectra, which indicates no generation of new groups. Since the (r)GO content is low compared with that of SF, and some of their characteristic peaks are very close, it is difficult to analyze the (r)GO-SF interactions from the FTIR spectra. Therefore, the proportion of secondary structures within

the amide I region was quantified by means of deconvolution (**Fig. 2B**). As expected, the amount of β -sheet structures increased after ethanol treatment due to increased crystallization resulting from the transition of α -helix into β -sheet [66]. Indeed, it has been suggested that the mobility of the polypeptide chains is likely to be reduced as ethanol strips off the water shell around fibroin, resulting in an increased rate of β -sheet formation [67]. The amphiphilic properties of GO could maximize hydrogen bonding, polar–polar, and hydrophobic–hydrophobic interactions with SF, which is composed of polar random domains and hydrophobic β -sheets. Furthermore, electrostatic interactions and hydrogen bonding between SF and GO can stabilize crystal structures and facilitate the formation of fibril structures [68]. However, it has also been reported that the introduction of GO can confine SF crystallization, leading to a decrease in crystallinity and crystallite size, and the formation of new interphase zones [64] (i.e. an oriented amorphous zone and the interface zone between SF and GO due to interfacial interactions between the hydrophilic blocks of SF and the oxygen groups of GO [68]). Here, a similar proportion of the various secondary structures were quantified on the substrates at the different GO loadings (**Fig. 2B**), with a slight reduction in the β -sheet content (p-value non-significant) at increased GO inclusion. These results tend to agree with those by Zhang et al. [64]. A similar proportion of the secondary structures were quantified for the various samples after in situ post-reduction into SF/rGO (**Fig. 2B**), with no significant differences with regards to the crystalline β -sheet content similar to the work of Zulan et al. [37]. Overall, similar silk-based scaffolds with no major changes in the molecular structure of silk were manufactured regardless of the addition of (r)GO. This is indeed important to help decoupling the influence of GO and rGO in subsequent cell-based assays.

Further studies were carried out to understand the compositional distribution of the fibers, where the presence of GO and rGO was confirmed by Raman spectroscopy (**Fig. 2C**). The

385 Raman spectra of the GO-containing scaffolds displayed two prominent peaks at around 1330
386 cm^{-1} and at 1595 cm^{-1} , ascribed to the D and G bands respectively. The G band is characteristic
387 of sp^2 -hybridized C–C bonds in a two-dimensional hexagonal lattice, while the D band
388 corresponds to the defects and disorder in the planar carbon network [69]. For the case of the
389 rGO-containing scaffolds, the D band slightly retracted towards 1315 cm^{-1} , while the G band
390 did not revealed any changes. A characteristic band attributed to β -sheets [38] could be
391 observed at around 1670 cm^{-1} for unmodified silk, and the absence of graphene in these fibers
392 was demonstrated by the lack of the characteristic spectral bands. The D/G intensity ratio can
393 also be used as an indicator of the defects in the graphene structure. In this study, the intensity
394 ratio of the samples (**Fig. 2D**) was found to increase with the filler content, which can be
395 correlated to increased carbonaceous presence in the composites [38]. The intensity ratio
396 decreased for rGO-containing samples, which could be attributed to a decreased amount of
397 oxygenated groups or structural defects after the reduction process [70,71].

Fig. 2 Effect of the presence of (r)GO on the chemical structure of silk. **(A)** Representative FTIR spectrum of the various scaffolds showing the typical fingerprint of silk. **(B)** Quantitative analysis of the secondary structure composition of silk, before and after treatment, within the amide I region (1700-1600 cm^{-1}). **(C)** Representative Raman spectra of the scaffolds. **(D)** Intensity ratio of the D and G spectral bands.

3.4 Conductive properties

Electrical conductance is dependent upon various factors, such as hydration degree, solvent or doping agent presence [44]. In this study, rGO flakes act as high aspect ratio electroconductive fillers in biohybrid composite electrospun silk-based scaffolds. Well-dispersed flakes within the fibers can contribute to the formation of conductive networks, generating self-assembling conductive pathways by forming conduction anchor nodes throughout. These conductive

pathways facilitate and enhance electronic transport, along with protonic (e.g. molecules of bound water and amino acid side groups of proteins [72]) and ionic (e.g. electrolytes from the medium; the conductivity of cells is ionic in nature) currents throughout the substrate. Cell-cell communication in electrically sensitive tissues can be enhanced this way. For instance, it has been shown that hydrogels synthesized in combination with multi-walled carbon nanotubes [56] or PEDOT-PSS [73] can act as internal enhanced electrical systems to promote the cell response. In both studies, cell-cell communication between cardiomyocytes was facilitated by enhancing the overall substrate's conduction and inducing cell elongation and unidirectional cell alignment with improved coupling and beating.

Fig. 3 Conductivity of electroactive SF/rGO scaffolds. Quantitative analysis of the conductivity properties of the scaffolds (n=4 per type) in the dry state, in the hydrated state and in the dry state after one-day hydration. Differences between the experimental groups were analyzed by two-way ANOVA with Tukey's test. n.s non-significant, *p<0.05, **p<0.01, ****p<0.0001.

Conductivity of SF/GO (Fig. S4) and electroactive SF/rGO scaffolds (**Fig. 3**) was estimated with a 4-point probe station. As expected, low conductivities were recorded on SF/GO scaffolds. Some differences were observed following addition of GO, though, in agreement

with a previous study on protein-based hydrogels with GO incorporation [74]. Reduced GO offers higher electrical conductivity in comparison to GO for applications where tuning electrical properties is the main target. The presence of rGO increased the overall conductivity up to $\sim 4 \times 10^{-5} \text{ S cm}^{-1}$ in the dry state, and peaked at $\sim 3 \times 10^{-4} \text{ S cm}^{-1}$ in the hydrated state. This was a synergic effect provided by both the substrate (electronic and protonic conductivity) and the electrolytes of the medium (ionic conductivity), and differs from the trend previously reported by Martins et al. where the conductivity of a carbon nanofiber/chitosan scaffold was dominated by the aqueous media and decreased upon hydration [75]. However, even in those cases where the ionic conductivity provided by the aqueous environment could dominate over substrates of lower inherent conductivity [75], local changes in conductivity attributed to the electroconductive/active moiety may directly affect the properties of cells by altering their resistance or capacitance. Hydrated protein-based materials [5,76–79], in this case silk fibroin [80], can also be considered as proton conductors. Water-mediated proton hopping takes place due to peptide and peptenol flip-flopping [80], and is known as the *Grotthuss mechanism* [5]. The electroactive SF/rGO scaffolds maintained their original conductivity in the dry state after one-day hydration.

On the other hand, rGO offers limited conductivity due to the presence of defects on the carbon backbone and the small flake size achieved during processing. Furthermore, high conductivity is subjected to achieving percolation threshold and a successful dispersion of the flakes within the polymer matrix. Although the conductivity exhibited here for most SF/rGO samples (dry/hydrated states) may fall short [81], it has been reported that a range 1×10^{-6} to $8 \times 10^{-5} \text{ S cm}^{-1}$ is sufficient for conducting electrical regimes in vivo [82,83]. The conductivities exhibited here in the dry/hydrated state fall within such range and exceed it, and could even be

further tuned to match or even surpass native tissues' conductivity by incorporating pristine graphene [84].

3.5 Neuronal cell viability, metabolic activity and proliferation

Neuronal-like cells were seeded onto all sample groups and their viability, metabolic activity and proliferation profiles evaluated. A viability assay was used to assess potential cytotoxicity of the different scaffolds, as well as evaluate attachment and survival of NG108-15 cells. Representative confocal micrographs and semi-quantitative analysis of viable and non-viable cells are shown in **Fig. 4** and Fig. S5. In all substrates, few dead cells were observed, with the great majority being alive (**Fig. 4A**). The percentage of viable cells after 7 days of culture was >95% for all scaffolds (**Fig. 4B**). No major differences in the ratio of viable cells were observed among the different substrates and the controls, demonstrating that the presence of GO or rGO in the system up to 10% wt. does not induce any major cytotoxic effects *in vitro*. During the growth phase, cells were observed to present round shapes with limited processes along their surface and grew clustered together in colonies. This is typical of NG108-15 cells and may have been further promoted by the randomly distribution of the fibers.

Fig. 4 In vitro cellular viability, metabolic activity and proliferation of NG108-15 cell-laden scaffolds over 7 days of culture: glass coverslip (CV), silk fibroin (SF), 1% GO/SF (SF/1G), 5% GO/SF (SF/5G), 10% GO/SF (SF/10G), 1% rGO/SF (SF/1R), 5% rGO/SF (SF/5R) and 10% rGO/SF (SF/10R). **(A)** Representative confocal micrographs of cells labeled with calcein AM (green) and ethidium homodimer-1 (red) as markers of cell viability; scale bars at 250 μm . **(B)** Semi-quantitative analysis of the percentage of viable and non-viable cells after 7 days in culture ($n=2$ scaffolds per type). **(C)** Metabolic activity relative to dsDNA content ($n=4$ scaffolds per type) and **(D)** cellular proliferation ($n=4$ scaffolds per type) of undifferentiated cells. Differences between the experimental groups were analyzed with two-way ANOVA with Tukey's test. n.s non-significant, $*p<0.05$, $**p<0.01$, $****p<0.0001$.

Many studies have investigated the toxicology profiles of graphene-family nanomaterials *in vivo* following different administration routes, as reviewed in [85]. Toxicology-mechanisms *in vivo* of graphene-family nanomaterials may include oxidative stress, DNA damage or inflammatory response amongst others. However, these are all largely influenced by the dose/concentration, lateral dimension/size, surface structure or functionalization degree, always leading to inconsistent results amongst different studies [85]. Their excretion and clearance from the body vary among different organs, but renal and fecal routes appear to be the main elimination routes [85]. Most experiments to date have focused on assessing toxicity in the lung and liver, and toxicity in the peripheral nervous system deserves more attention. Recent *in silico* toxicity screening studies of graphene-containing silk-based materials confirm skin sensitization due to the presence of conjugated dienes in their structures [86], but at much higher loading concentrations (> 10% wt.) than the ones used in the current study.

The metabolic activity of NG108-15 cells relative to the number of cells in culture is presented in **Fig. 4C**. Cells exhibited greater metabolic activity on substrates containing GO and rGO compared to unmodified silk, tending to increase with filler content. By day 5, cells grown on electroactive SF/rGO samples were metabolically more active than those grown on SF/GO, while by day 7 the trend was opposite, which might suggest cell confluency. The metabolic activity results were well supported in terms of cellular proliferation on the various substrates over time (**Fig. 4D**), with the SF/rGO scaffolds accelerating cellular proliferation to the greatest extent. These differences in cellular behavior could be directly attributed to the presence of electroactive rGO as opposed to GO.

Nevertheless, surface roughness and protein adsorption can also affect cell-material interactions [87], leading to changes in cellular adhesion, proliferation and biosynthesis of ECM components. Addition of (r)GO into the silk system led to a significant increase

501 (p<0.001) in surface roughness for all samples tested (**Fig. 5A**, and Fig. S6) compared to
502 unmodified silk. Similarly, previous reports have shown increased surface roughness at
503 increased graphene [84], carbon nanofiber [88] and carbon nanotube [89] content within
504 various composites. Such changes may lead to regionally organized micro/nano- topographies
505 that can contribute to micro/nano- structure-induced cellular interactions [89]. Non-significant
506 differences in surface roughness were observed between the GO and rGO counterparts prior to
507 and after post-reduction. The ability of the scaffolds to adsorb protein was also quantified (**Fig.**
508 **5B**). Protein adsorption tended to increase especially at increased (r)GO loading compared with
509 unmodified silk. This is in agreement with other studies [88,90] that have correlated an increase
510 in protein adsorption with an increase in surface roughness. However, regardless of the GO
511 and rGO counterparts presenting similar surface roughness, lower amounts of laminin were
512 adsorbed on the rGO-containing samples. These results are in line with previous findings that
513 have demonstrated that GO shows greater adsorption of proteins on its surface compared with
514 rGO [91–93]. Therefore, while some studies have suggested that surface roughness and protein
515 adsorption can be more vital traits over the electronic configuration of the scaffold [6], the
516 tendency of electroactive SF/rGO to outperform SF/GO substrates in terms of the cellular
517 response (**Fig. 4C-D**) could be attributed here to the endogenous electrical regimes induced by
518 rGO.

Fig. 5 Surface roughness and protein adsorption on the scaffolds. **(A)** Quantitative analysis of the surface roughness parameters (Sq – root mean square height, and Sa – average mean height) of the scaffolds (n=2 per type). **(B)** Quantitative analysis of laminin adsorption on the scaffolds (n=3 per type) after 2 h incubation. Differences between the experimental groups were analyzed by two-way ANOVA with Tukey’s test. n.s non-significant, **p<0.01, ***p<0.001, ****p<0.0001.

3.6 Neuronal differentiation

Neurite outgrowth, axonal elongation and the formation of neuronal circuits are essential for the nerve tissue repair and regeneration process. Neurite outgrowth can be guided by physical cues in the substrate, but also directed by electrical regimes. Inspired by the cell metabolic activity and proliferation results, NG108-15 neuronal-like cells were grown on the various scaffolds in serum-deprived conditions and immunolabeled with β -tubulin III as a marker for neurite outgrowth and differentiation (**Fig. 6**). β -tubulin III is a marker expressed in neuronal cell bodies, dendrites, axons, and axonal terminations. Cell-laden samples in serum-supplemented medium served as negative controls of differentiation (Fig. S7), with cells

growing very clustered together without neurite extension. Representative micrographs of the various cell-laden substrates after induced differentiation are shown in **Fig. 6A**. Neurite outgrowth on all substrates could be clearly observed after immunolabeling. Neurite length was quantified for each scaffold type (**Fig. 6B**). Larger neurite lengths were measured on SF/GO scaffolds compared to unmodified silk, but these differences were not significant. Further analysis revealed maximum neurite extensions of 207, 181, 212, 207, 192, 164, 258 and 151 μm for coverslip, SF, SF/1G, SF/5G, SF/10G, SF/1R, SF/5R and SF/10R, respectively. This suggests generation of longer neurites for GO containing scaffolds in comparison to unmodified silk, and tended to reduce for the rGO counterparts. The largest measurement was reported for electroactive SF/5R, though. The average number of neurites expressed per NG108-15 neuronal cell body was estimated at ~ 1.4 (**Fig. 6C**), in line with what has been previously reported using the same NG108-15 cell line on other scaffolds [94,95]. The average number of neurites per neuronal cell body decreased at increased GO content, while no differences were observed among the rGO/silk composites.

Fig. 6 Differentiated NG1081-15 cells on the scaffolds after 5 days of culture: glass coverslip (CV), silk fibroin (SF), 1% GO/SF (SF/1G), 5% GO/SF (SF/5G), 10% GO/SF (SF/10G), 1% rGO/SF (SF/1R), 5% rGO/SF (SF/5R) and 10% rGO/SF (SF/10R). **(A)** Representative confocal micrographs of differentiated cells immunolabeled with β -tubulin III (green) and cell nuclei counterstained with DAPI (blue); scale bar at 75 μm . **(B)** Semi-quantitative analysis of the average neurite extension on each scaffold (n=2 per type). **(C)** Semi-quantitative analysis of the average number of neurites per neuronal cell body for each scaffold (n=2 per type). Differences between the experimental groups were analyzed with one-way ANOVA with Tukey's test. n.s non-significant, *p<0.05.

Future studies should investigate the degradation properties of the produced scaffolds. Silk fibroin is not expected to degrade within the 7-day culture period in DMEM medium

undertaken in this study since it has extensive beta-sheet formation. Minimal solubilization and degradation behavior of regenerated silk fibroin has previously been reported *in vitro* up to 12 weeks [96] in DMEM/PBS conditions. Degradation *in vivo* could be tailored upon the molecular weight of the regenerated silk fibroin (dissolution method), beta-sheet crystal content [97] and porosity of the scaffold [98,99] to match the degradation rate required for peripheral nerve repair.

4. Conclusion

Delineating the role of electrical conductivity in cell behavior is challenging, since it is difficult to manufacture scaffolds with similar physico-mechanical properties but different levels of conductivity, completely decoupling combined effects. Here, SF composite fibrous scaffolds were manufactured by incorporating GO at loading concentrations up to 10% wt., optionally followed by *in situ* post-reduction into electroactive SF/rGO. Presence of GO or rGO did not substantially influence the substrates in terms of the fiber size distribution, porosity, water sorption or protein secondary structure in silk. In contrast, surface roughness and protein adsorption capacity tended to increase with increasing filler content. Reduction treatment into SF/rGO greatly enhanced the electrical conductivity of the scaffolds, which was further promoted after hydration. Analog neuronal NG108-15 cells were able to adhere and survive on all samples. Metabolic activity and proliferation rates were enhanced with the presence of GO and were notably outperformed after reduction into electroactive SF/rGO. Furthermore, these conductive composites supported neurite outgrowth. While it is difficult to pinpoint the parameter responsible for the differing cell responses observed between the GO and rGO counterparts, the inherent electrical regimes conferred by rGO are an important trait over differences in surface roughness or protein adsorption, and further investigation should be warranted. Future studies can look at screening these substrates with other neuronal and

Schwann cells, or in co-culture conditions. In addition, promotion of neurite outgrowth could be further investigated in combination with aligned fibrous substrates and direct electrical stimulation.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

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