



Collagen/pristine graphene as an electroconductive interface material for neuronal medical device applications

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ABSTRACT

The growing clinical demand for electrical stimulation-based therapies requires the development of novel conductive biomaterials that balance conductivity, biocompatibility, and mechanical performance. Traditional conductive materials often induce scarring, due to their stiffness and poor biocompatibility, presenting challenges to their clinical translation. To address these issues, we report the development of an electroconductive pristine graphene-based (pG) composite material for central nervous system applications, consisting of type I collagen loaded with 60 wt% pG to yield conductivities (~1.5 S/m) necessary for efficient electrical stimulation. Neurons and glial cells grown on composite films exhibited robust growth, and glial cells exhibited no change in inflammatory markers. Electrical stimulation of primary neurons on the composite enhanced neurite outgrowth, cellular viability and morphology compared to collagen controls. Finally, we demonstrated the versatility and potential applications of the composite material for neuronal medical device applications by fabricating a range of conductive, neural-interfacing structures, including porous scaffolds, microneedle arrays, and 3D-printed circuits for bioelectronics. These results show that CpG composites form a versatile neurotrophic platform that balances biocompatibility and physiologically relevant conductivity with robust mechanical properties that allow for the production of a range of next-generation neuroprosthetic devices.

1. Introduction

As our understanding of central nervous system (CNS) function has rapidly evolved over the past 50 years, the neural interfaces used in medical research and therapeutics to interrogate and/or influence CNS electrical activity patterns have largely remained unchanged. As such, there is an unmet demand for versatile electroconductive materials,

capable of interfacing safely and consistently with neural tissues, that will enable the development of the next generation of neural medical devices. The inclusion of charge-carrying nanomaterials presents the possibility of imbuing a wide array of polymer materials with conductive properties, thus presenting an attractive alternative to existing materials used as neural interfaces (e.g., metal electrodes). We present a nanocomposite of pristine graphene (pG), a highly conductive

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nanomaterial, and type-I collagen, a naturally occurring organic polymer, as a versatile, neurocompatible alternative to commonly used electrode materials. We demonstrate its capacity to effectively enhance the response of neurons to electrical stimulation, and its versatility for application to different neural medical device designs.

With their advent in the early 1960's, the fundamental component of many neural interfacing devices is a conductive electrode, with stimulatory or sensing features and which allow for the connection of the organic tissue to a functional electronic device such as a piezoelectric nanotransducer [1], triboelectric nanogenerator [2,3], external power supply, or sensing device, without disturbing normal tissue function. Many neural interfaces employ metal-based electrodes, which have ideal conductive properties, but the disparity in their physicochemical properties compared to the softer neural tissues often leads to glial cell-induced scarring and long-term inflammation. This 'foreign body response' is driven in its early stages largely by neuron-supportive astrocytes and immune-surveillant microglia, and leads to post-implantation microhemorrhage and scarring, which can limit long term performance [4,5]. To overcome this issue, recent electrode designs look to incorporate softer conductive materials which more closely mimic the physicochemical properties of neuronal tissue [4,6–9], improving the neurocompatibility and subsequent implant lifespan.

Foremost among the candidates for these new softer electrodes are composites with conductive nanomaterials [7,10–12], or conductive polymers [12–16]. Carbon allotropes are particularly attractive due to their unique mechanical, thermal, optical, and electrical properties [10, 17]. Among the various carbon allotropes, the graphene family, consisting of pristine graphene (pG), graphene oxide (GO) [18], and reduced graphene oxide (rGO) [19], have been identified as strong potential candidates for application at the neural interface [6,20–22]. pG in particular possesses excellent electrical conductivity [17,23], strength, and processability [23], and thus, has been identified as a strong potential candidate for application at the neural interface [6,20]. These properties result from its 2D molecular structure, comprising a one atom thick layer of sp^2 hybridized carbon atoms, arranged in a planar honeycomb structure [5]. However, its potential as a building block for electroconductive devices has been marred by challenging production processes [11,24], particularly when aiming to produce biocompatible pG [25]. Graphene oxide (GO) consists of a graphene basal plane with plentiful surface oxides, which have the benefit of rendering the surface hydrophilic, but which also disrupt the basal plane of the resultant nanosheets, resulting in a sharp decrease in the conductivity compared to pristine graphene [26]. Reduced graphene oxide (rGO) is synthesized by the reduction of graphene oxide to recover the delocalized π -electron structure present in graphene. The conductivity is improved compared to GO, however often still lower than pristine graphene, due to defects and incomplete removal of functional groups on the basal plane [27].

Despite the superior conductivity of pG [17,23] the difficulties in producing consistent quantities of high quality, biocompatible pristine graphene compared to GO or rGO has slowed research into its biological application [25,28], and has led to inconsistent reports regarding its neurocompatibility [29–32]. However, recent reports indicate that the biocompatibility can be enhanced by controlling crystalline quality [31], modifying surface chemistry [33,34], and non-covalent surface functionalization, while retaining its favorable mechanical, thermal, optical and electrical properties [29,35,36]. Previously, we have reported enhanced bio-functionality of pG through complexation with biomolecules, both in suspension and in a substrate, while retaining its conductive properties [37–39]. By employing a high yield liquid-phase exfoliation protocol, using the biopolymer gelatin as a stabilizer [39], the use of toxic stabilizing reagents [25,40] is avoided, to produce graphene flakes that are stable in water and easily incorporated with other biopolymer-based matrices.

The lack of toxicity is crucial for the effective use of materials in any biological context and several different approaches and cell types have

been used to assess graphene biocompatibility [18,37,41–44]. Cells exposed to graphene suspensions [30,37] or seeded directly on pure GO or pG surfaces have been reported to induce cellular damage [45] and apoptosis [30]. Neurons also show altered excitability when directly exposed to pG [21]. However, biocompatibility is often increased if the graphene surfaces are first functionalised by coating with a trophic biological substrate [41,46–49], avoiding direct cellular contact and allowing for more robust growth [36]. A similar strategy has also been employed to enhance neuroprosthetic device compatibility *in vivo*, with the electroconductive surfaces often coated or encased in a biological substrate [44,50–52], but this often requires multistep fabrication processes. Developing an innately biocompatible and electroconductive composite that does not require further functionalisation steps, yet is versatile enough to produce complex geometries, is an attractive solution.

We hypothesized that a combination of collagen and pG (CpG) could yield a nanobiocomposite with the requisite conductivities necessary to interact with the central nervous system for neural interface applications [53]. Type-I collagen is an abundant extracellular protein in the human body and has been widely used for neuronal tissue engineering platforms [54–56], due to its high levels of biocompatibility with nervous system cells [50]. When pG is bound into collagen substrates it has been reported to be non-inflammatory [37,57] and supports the growth of directly seeded cardiomyocytes [38].

In this study the effect of pG loading on the properties of the resultant CpG nanocomposites was investigated. The resulting materials exhibited physiologically relevant conductivity and favorable mechanical properties, resulting in a soft, stable, and conductive material. Moreover, we investigated the neurocompatibility of the substrate to support the growth of several neuronal types, and the immuno-compatibility of the CpG composites focusing on both astrocyte and microglial responses, which are key regulators of the foreign body response in the CNS. All cell types exhibited healthy morphologies and glial cells exhibited cytokine release profiles indicative of non-inflammatory phenotypes - indicating that CpG exhibits both excellent neurocompatibility and immuno-compatibility. The ability of the CpG60% (60 wt% pG) nanocomposite to deliver functional electrical stimulation [58,59] to mouse primary cortical neurons (MPCNs) was next demonstrated. Cells stimulated for 7 days showed healthy morphologies, and enhanced neurite length and density. Finally, we show the versatility and potential applications of the composite material by fabricating a range of conductive, neural-interfacing structures – specifically a lyophilized macroporous bioscaffold, a microneedle array, and a conductive bioink. Together, these data show that CpG nanocomposites are versatile materials with enhanced neurocompatibility and conductivity, enabling effective delivery of electrical stimulation for a wide array of neural applications.

2. Results and discussion

2.1. Production and characterization of films of type I collagen and pristine graphene

To assess the physical characteristics of the collagen/pristine graphene (CpG), candidate composites were dry cast to produce homogeneous films. The effect of increasing pG concentration on the mechanical properties of each candidate composite was assessed by performing tensile tests on PBS-hydrated films, to reproduce the conditions found *in vivo*. Young's modulus (E) of the composite films initially decreased after graphene addition (10–30 wt%) (Fig. 1A). This may be due to the effect of pG disrupting the interaction between individual collagen fibrils at high graphene loadings, leading to weakening of the material [39,60]. The maximum elastic modulus, ultimate tensile strength, and maximum strain are available in the Supplementary Information (Sup. Fig. 1). Maximum elastic modulus and ultimate tensile strength are both significantly lower for the CpG composites than they are for collagen. The maximum strain is significantly lower in the CpG60% composite,

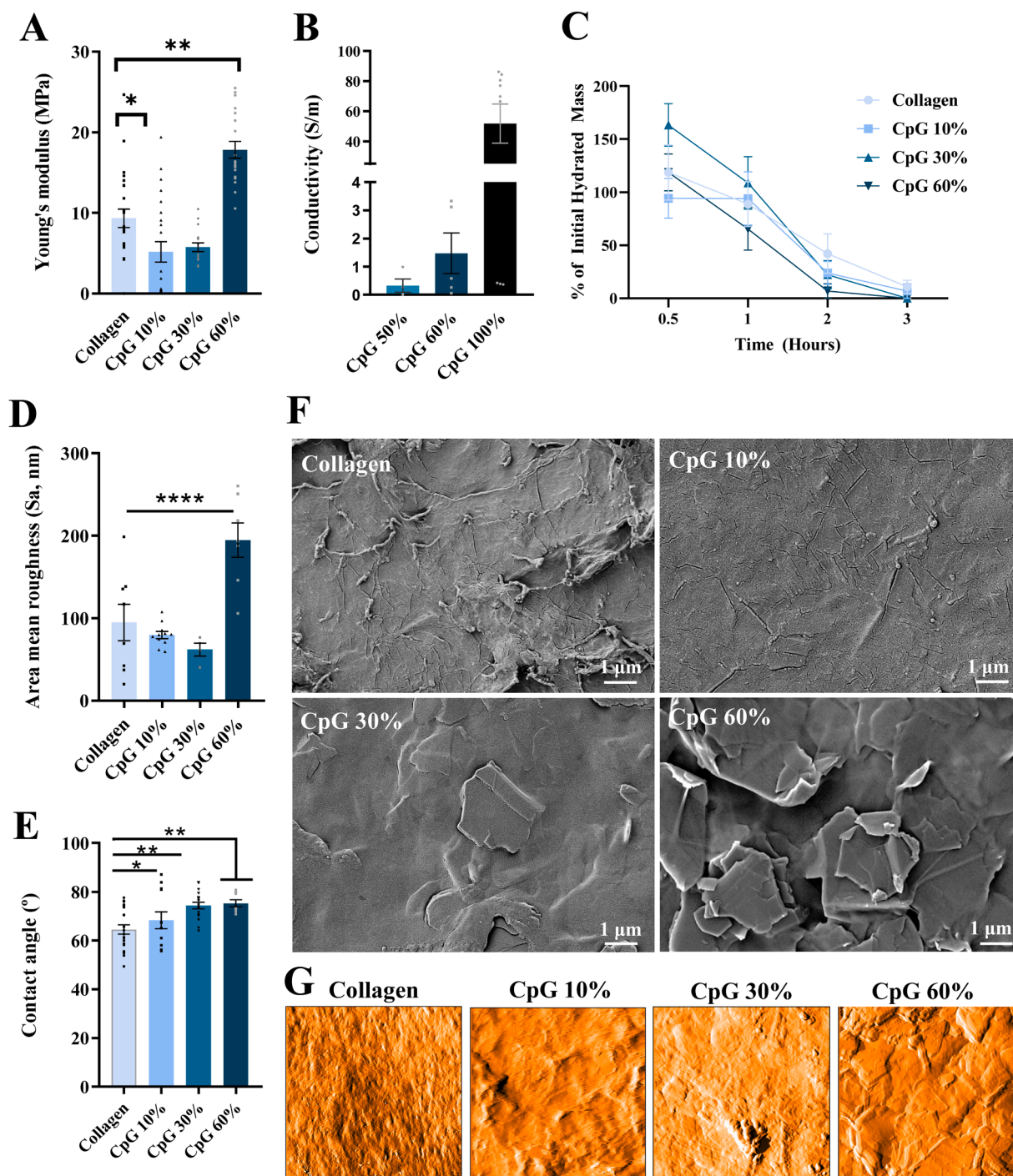


Fig. 1. Bulk physical property characterization of collagen/pristine graphene (CpG) composite films. (A) Assessment of the Young's modulus of the hydrated films, as a function of pG loading and obtained by stress-strain measurements, showing the robust mechanical properties of CpG. (B) Conductivity measurements in dry state, showing the composite reaching the percolation threshold as a result of increasing pG loading. CpG 100% represents a vacuum-filtered, polymer free network of graphene. (C) Degradation testing of the films with different pG loadings using the enzyme collagenase (at 37 °C) revealed that pG loading has no effect on degradation rates compared to the collagen controls. (D) CpG film area mean roughness showed significant increase in surface roughness on CpG60% compared to other tested films. (E) Contact angle measurements performed with deionized water droplets on the different composites showed a small but significant increase with higher pG loadings. (F) SEM imaging of the film surfaces revealed an increase in irregular shaped surface profiles with higher pG loadings. Scalebars = 1 μm. (G) AFM images derived from 10×10 μm z-axis scans for increasing graphene loadings also showed an increase in surface irregularities with higher pG loadings. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

indicating a decrease in the ductility of the material. Nanomaterial reinforcement at low loadings is explained using the Rule of Mixtures for nanocomposites, while at the high loadings used in our study, the deterioration in mechanical properties, and thus deviation from the Rule of Mixtures is explained by increasing filler aggregation [39,60,61].

However, Young's modulus of the 60 wt% composite was significantly higher ($E = 17.8$ MPa, $p < 0.001$) than the collagen controls (Fig. 1A). It is unclear where this reinforcement arises from, since the mechanical properties should deteriorate at high loadings, as detailed above. It may arise due to the unusual fibrillar nature of collagen compared to other

polymers, which may cause deviation from the expected mechanical properties at higher graphene loadings [62]. Taken together, these data show that the mechanical properties of all CpG composites are closer to the stiffness of CNS tissues (1–100 kPa) [63] compared to that of traditional CNS electrode materials (>100 GPa) [64], and on the same order of magnitude as collagen, which has been used extensively in neural tissue engineering [65]. The lower stiffness of the CpG films compared to traditional electrode materials is an important material property, as exemplified by recent work, which has shown that matrix-based materials interact with glial cells in a stiffness-mediated manner [66]. The flexural modulus and the electrical performance of the material under repeated deformation may be relevant, since biomaterials can be subject to specific tensile, compressive, and flexural stresses depending on the location of the implant in the CNS (e.g. cortex versus spinal cord). However, these parameters depend largely on the design and location of the implant. The versatile processing capabilities of the CpG60% material would allow the fabrication of devices with a range of flexural moduli, to fit the desired application.

Preserving the electrical properties is a key parameter for any electroconductive material [67] designed for biological applications, so we next determined the conductivity of all CpG candidates in the dry state, with the percolation threshold being reached at approximately 50 wt% pG (Fig. 1B). The highest conductivity with physically stable films was achieved at 60 wt% loading (~1.5 S/m), rendering the composite sufficiently conductive for nervous tissue [53], and higher than reported previously for graphene stimulatory composites [42]. It is likely that at these higher loadings, conductivity trades off with strength, as decreasing quantities of polymer binder in a composite will likely lead to the strength of the composite relying on the relatively weak bonds between graphene flakes [39,60].

We next assessed the thermal and physiological degradation properties of the composites, to determine their stability in long-term physiological conditions. Thermogravimetric analysis demonstrated a trend towards higher thermal stability with increasing graphene loadings (Sup. Fig. 1D), indicating higher thermal stability compared to collagen alone, and confirming the ability of the composite to undergo dehydrothermal (DHT) treatment at 105 °C without compromising the structural integrity of the material. To assess the stability and degradation capacity of the composite films under accelerated physiological conditions, each film was subjected to rapid enzymatic degradation using collagenase (0.1 mg/mL) at 37 °C. Significant loss was seen in all groups after one hour, and total loss registered after 3 h, with no difference between all groups, indicating that pG loading does not impact the enzymatically induced breakdown kinetics of collagen in the films (Fig. 1C). This finding indicates that the CpG composite material shares a similar degradation profile to collagen controls.

Material surface properties are critical for appropriate material-cell interaction [68]. The topography of film composites with the different pG loadings were investigated using scanning electron microscopy (SEM) (Fig. 1F). In all composites, pG flakes visibly protruded from the surface, with flakes forming larger irregular 3D features on CpG60% films. To corroborate these observations, atomic force microscopy revealed similar surface topographical changes (Fig. 1G), with CpG60% showing increased area and minimum line roughness (Fig. 1D and Sup. Fig. 1G). Next, to determine the correlation between film hydrophobicity and topography, surface contact angles were assessed, revealing that higher pG loadings lead to significantly higher contact angles (~74° c.f. 65° for collagen), denoting a decrease in surface hydrophilicity (Fig. 1E). The associated changes in surface roughness and hydrophobicity are known to affect cell attachment and biocompatibility [69,70] and may have a positive impact on the ability of the substrates to support CNS cell growth [71].

2.2. Characterization of neuronal and glial cell response to CpG-based films

Next, to assess the biocompatibility of the different CpG composites, human SH-SY5Y neurons were seeded directly onto the film surfaces for 3 days, and the change in metabolic activity, DNA content and cell morphology assessed (Fig. 2A, C). Neurons examined at 1 and 3 days after seeding grew robustly, with no difference in metabolic activity and DNA content (Fig. 2B, C) between the film formulations, indicating the formation of a stable population of neurons on the film. The cells exhibited typical neuronal morphology, usually extending multiple long neurites from the cell body (Fig. 2A). To verify the ability of the composites to support the growth of different neuronal types, motor neurons (NSC-34 line) were also cultured on the films and exhibited similar robust growth, with no difference seen between the controls and composite formulations (Sup. Fig. 2). To further investigate the long-term impact of CpG substrates on human neuronal survival and growth, induced pluripotent stem cell-derived (iPSC) neurons were cultured on collagen and CpG60% films for 15 days. Growing neurons on both films (Fig. 2D, E) extended multiple long neurites that formed dense meshwork of processes between the neurons, with the CpG composite exhibiting no difference to collagen controls.

While several studies have assessed neuronal growth on graphene substrates, many use secondary biomaterial coatings [46–49] to improve surface biocompatibility, and may inadvertently shield the neurons from direct interaction with the material. Where neurons have been seeded directly onto graphene immobilised surfaces [18,36,72] the material supports their growth but is a poorer substrate compared to ECM-coated graphene [36]. In contrast, the ability of CpG60% to facilitate robust neural cell growth in both SHSY-5Y and NSC34 neurons and young iPSC-derived neurons, and enhance primary neurite outgrowth compared to collagen controls (see Fig. 5A–C), suggests that graphene-ECM composites provide a better environment for neuronal colonisation and growth, perhaps mediated through the irregular surface profiles (see Fig. 1F) which are known to strongly influence cell attachment and growth [73]. These data clearly demonstrate that the CpG60% composite supports long term survival and growth of young human-derived neurons.

The CNS is particularly sensitive to prosthetic implants and foreign materials, and rapidly generates a foreign body response (FBR) [74] to produce dense scar tissue around the implant [4,5,75]. This process is driven by the reactions of both immunoresponsive microglial cells and astrocytes, the major supportive glial cell type in the CNS [76], which form reactive phenotypes that can reciprocally signal to each other through cytokine release to start developing the dense glial scar layers [77,78]. Therefore, to assess whether the composites may induce ‘injury-reactive’ pro-inflammatory phenotypes we next assessed if the CpG composites impacted the growth of microglial cells (Fig. 3A–C) and (Fig. 3D–F). Microglial cells seeded on all composite films exhibited a robust >3-fold increase in DNA between 1 and 3 days after seeding (Fig. 3B; T1 vs T3, $p < 0.0001$), with cells possessing circular morphologies, typical of this cell type when grown on 2D surfaces [79]. Similarly, human-derived astrocytes seeded on the films exhibited the same prolific (>3-fold) growth with significant increases in DNA content in all groups between days 1 and 3 ($p = 0.003$) (Fig. 4C), with the growing cells forming typical long dense parallel arrays of cells with elongate morphology without evidence of toxicity [80]. A small number of studies have focused on astrocyte responses to graphene *in vitro* [81, 82] and *in vivo* [21,80]. As was found in the present study, in general graphene-containing substrates do not appear to adversely affect astrocyte viability and proliferation [83]. However, astrocyte interaction with pure forms of graphene such as uncoated flakes can lead to cellular internalization [84] and induce changes in homeostatic function [82,84,85]. In contrast, we found no evidence of cellular stress with cell morphology, proliferation and metabolic activity similar to cells grown on collagen films, indicating that the composites provide a stable

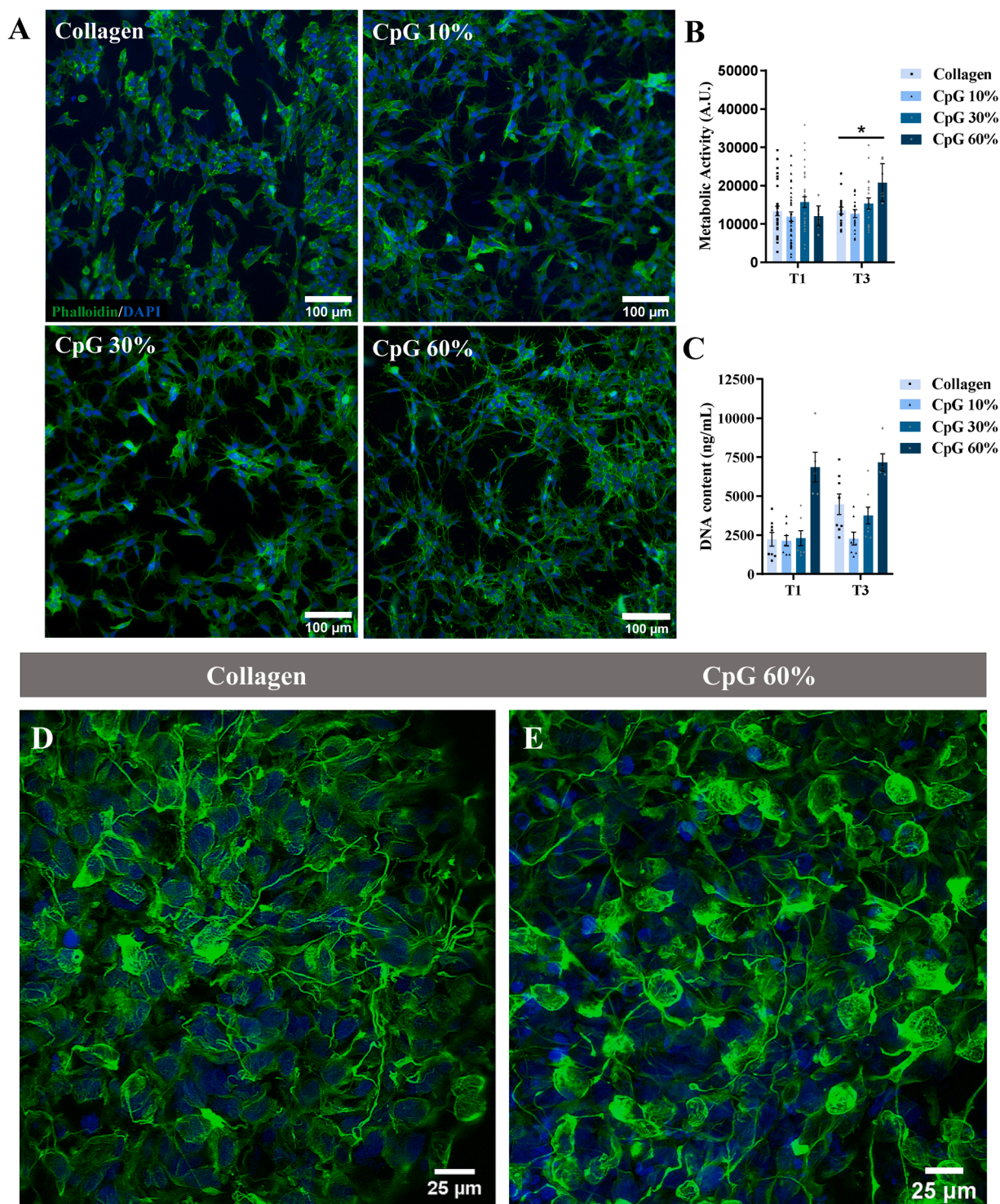


Fig. 2. Characterization of neuro-compatibility of collagen/pristine graphene films. (A) Phalloidin (actin = green) and DAPI (nuclei = blue) staining reveals the full morphology of SH-SY5Y neurons grown for 3 days on the collagen (control) and different CpG composites showed no difference in morphology, with all neurons extending long neurites across the film surfaces. (B-C) Comparison of metabolic activity (B) and DNA content (C) at 1 and 3 days after seeding indicated that neurons readily colonized all film surfaces, in particular at higher CpG loadings. (D-E) β -tubulin III immunostaining of iPSC neurons (counterstained with DAPI (blue)) cultured for 15 days on collagen (D) and CpG60% (E) films equally generated dense networks of neurons that extended multiple long neurites. Scalebar in A = 100 μ m, in D, E = 25 μ m. * $p < 0.05$.

trophy environment for astrocyte growth.

Since both cell types can reciprocally stimulate each other through cytokine-mediated signaling pathways [86] in the diseased and injured CNS [87], their cytokine expression profiles are robustly indicative of pro-reparative (e.g. IL-10) or pro-inflammatory (e.g. IL-1 β and IL-6)

activation states [86,88,89]. Therefore, we next investigated the effect of increasing graphene content on the release of key immunomodulatory cytokines from both cell types. For microglia, the release of pro-inflammatory IL-1 β and pro-reparative IL-10 (Fig. 3C) from cells grown on films was sequentially measured at 1, 2 and 3 days after

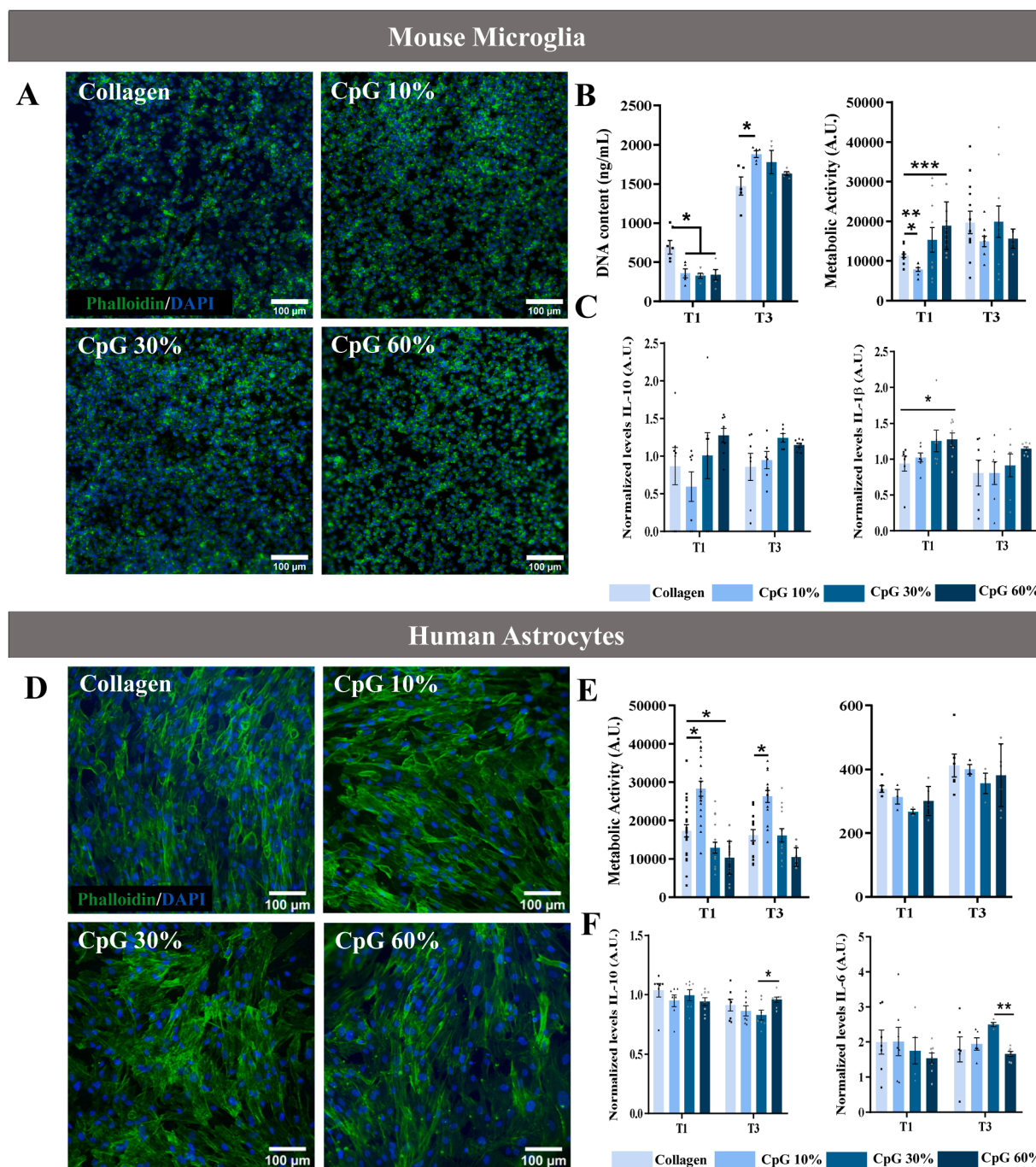
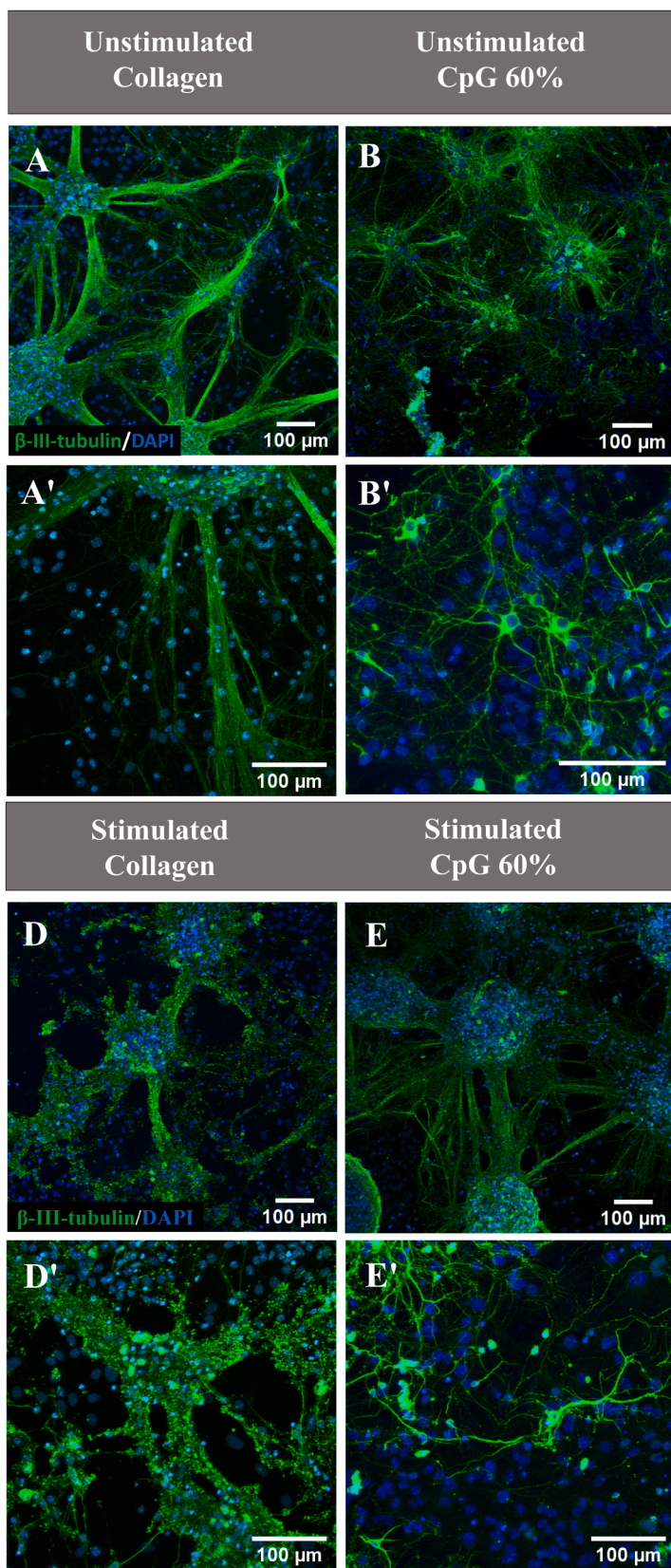


Fig. 3. Characterization of glial cell compatibility of collagen/pristine graphene films. Microglia (A-C) and astrocytes (D-F) were grown for 3 days on CpG films to assess the effect of CpG composites on their growth, morphology, and pro-/anti-inflammatory cytokine expression profiles. Microglia showed typical circular morphologies when grown on the collagen and composite films (A) and exhibited metabolic activity and DNA content changes (B) indicative of rapid colonization across the film surface. Similarly, astrocytes exhibited typical elongate morphologies with long processes (D) and changes in metabolic and DNA content indicative of dividing cells (E). ELISA based analysis of the pro-inflammatory cytokines IL-1 β and IL-6 released by microglia (C) and astrocytes (F), respectively, and the anti-inflammatory cytokine IL-10 released by both cell types indicated no difference in proinflammatory cytokine release for either cell type, and a trend for increased release of IL-10 from astrocytes, in particular cells grown on CpG60% films (F). Values shown are concentration levels (pg/mL) of test conditions normalized to negative controls on tissue culture polystyrene plates. Scalebar = 100 μ m. * p < 0.05, ** p < 0.01, *** p < 0.001.

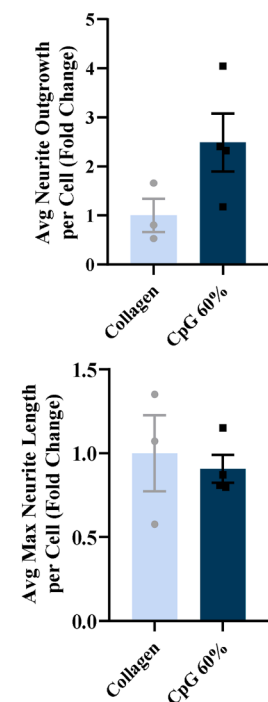
seeding. For both cytokines there was no significant change in levels between all groups, suggesting that all film types encouraged a non-polarized ‘resting’ phenotype, and are similar to reports of primary immune cell behaviour when exposed to different graphene-based structures [42,90,91]. We then investigated the release of pro-reparative IL-10 and pro-inflammatory IL-6 (Fig. 3F) from human astrocytes grown on the films over the same 3-day period. In a similar

pattern to the cytokine expression seen in microglia, there was no overall significant change in the levels of both cytokines, but there was a trend for reduced levels of IL-6 and increased IL-10 release in CpG60% films compared to other composites.

When taken together, these data show the ability of both glial cell types to proliferate and colonize the film surfaces, which when coupled with no change in inflammatory IL-1 β and IL-6 cytokine release and a



C Unstimulated



F Stimulated

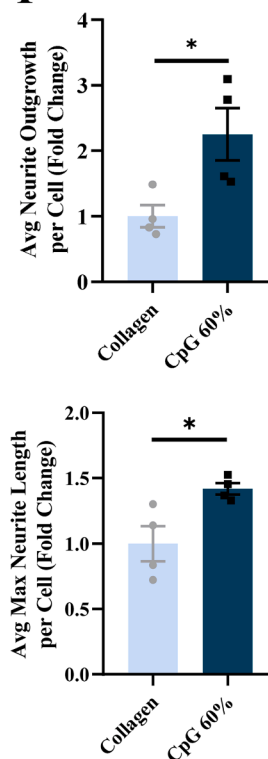


Fig. 4. CpG films support long term primary neuron growth and enhance neurite outgrowth with external electrical stimulation. (A-B) DAPI-stained and β -tubulin III immunostained mouse primary cortical neurons grown directly on the surface of collagen (A) and CpG60% (B), seen at higher power in (A') and (B'), respectively, exhibited robust neurite outgrowth on both substrates. (C) Analysis of neurite outgrowth and maximum neurite length in unstimulated mouse primary cortical neurons indicated no significant difference between collagen and CpG films. (D) Stimulated neurons grown for 14 days on the collagen films showed a greater fraction of neurons exhibiting cell stress, seen as beading and poor neuronal morphology (D') whereas neurons grown on CpG60% films (E) showed robust neurite outgrowth (E') and morphologies typical of healthy neurons. (F) Neurons grown and stimulated on CpG60% films showed significant increases in neurite outgrowth and maximum neurite length compared to collagen controls. Scalebars A-E' = 100 μ m. * $p < 0.05$.

trend for increased IL-10 expression in astrocytes indicates that both cell types rapidly grow on the films, without inducing inflammatory phenotypes. This is significant, as graphene-mediated cellular stress is known to affect proinflammatory cytokine release [92], which was not

found for the cells grown on the CpG composites. Rather, the trend for increased astrocyte release of IL-10, a strong modulator of inflammatory cytokine production in microglial cells [93], suggests that higher graphene content may encourage non-inflammatory astrocyte phenotypes.

These data thus directly address a primary concern of many electroactive materials, i.e. adverse glial cell responses, and indicate that CpG60% in particular is a strong candidate for a bio-interfacing conductive substrate. There are of course other factors specific to the *in vivo* foreign body response that cannot be fully accounted for by *in vitro* testing, such as the size, geometry, and electrochemical properties of the implant [94–96]. Our use of 2D films to monitor CNS cell responses is a common approach taken by many other studies [36,41,48], as it simplifies the assessment of cell responses to materials and minimizes the ability of irregular surface features and geometries to influence cell growth [70]. The evidence from *in vivo* studies indicating some tolerance of graphene flakes [32] and that collagen coated electrodes induce minimal scar development [50,52], when coupled with the rigorously demonstrated biocompatibility of the CpG composites using four different types of neurons (two cell line, one iPSC, one primary, Fig. 4A,B) and two CNS glial cell types (above), is strongly indicative of a well-tolerated biocompatible material composite. In addition, the robust growth of primary and iPSC-derived neurons in direct contact with the composite over two weeks is also indicative of the potential of this material for safe longer-term interaction with neuronal circuitry.

2.3. Assessment of the synergistic effect of electrical field stimulation and conductive CpG films on neural cell morphology and function

With the CpG60% nanocomposite shown to be neurocompatible and possess electroconductive properties potentially suitable for neuronal stimulation, we next assessed its ability to support primary neuron isolates and encourage neuronal process growth under electrostimulation. Previous electrostimulation studies demonstrated the ability of laminin coated graphene films to support neural stem cell-derived growth [47, 49]. Therefore, we reasoned that primary neuron isolates grown directly on CpG60% films and subjected to electrostimulation should be capable of supporting the neurons and enhancing neurite outgrowth. Mouse primary cortical neurons (MPCNs), which have previously been used in electrostimulatory studies [97], were isolated and seeded on collagen (control) and CpG60% films and allowed to grow for 7 days. Thereafter, the neurons were stimulated for a further 4 h/day over 5 days (12 Hz, 9.8 ms, 200 mV/mm). These parameters, at the lower end of the long term neuronal electrostimulatory range [59] were chosen to avoid the creation of deleterious electrochemical species and field-induced cellular damage. While cells grew robustly on all films, the morphology of growing MPCNs on CpG60% films in both the unstimulated and the stimulated conditions (Fig. 4B, E) formed denser neuronal networks, and possessed longer neurites compared to neurons grown on collagen films (Figs. 4A, D, Sup. 4–6). In general, stimulated and unstimulated MPCNs grown on collagen films contained a greater fraction of stressed/degenerating neurons (assessed by beading on neurite processes [98]) compared to those grown in CpG60% films, despite collagen previously being shown to act as a suitable substrate for neuronal culture and *in vivo* CNS implantation [65,99,100]. This finding may be indicative of the beneficial effect of the rougher CpG60% film surface to support neuronal growth compared to the control collagen films. MPCNs grown without stimulation on both substrates extended long neurites that often formed a complex meshwork of processes, with no significant difference in length (Fig. 4C), although the neurons on CpG60% exhibited a near significant ($p = 0.0855$) increase in neurite length per cell. When subjected to electrical stimulation, however, cortical neurons on CpG60% films exhibited significant increases in average neurite outgrowth per cell and the average maximum neurite length when compared to stimulated neurons on collagen substrates (Fig. 4F). A similar increase was observed for the number of intact neurites per cell (Sup. Fig. 5B,C). These results clearly illustrate that CpG60% films support healthy growth of cortical neurons, and are similar to findings from studies that utilised primary neurons grown on ECM-coated graphene films [46,48] again indicative of strong composite biocompatibility. More importantly, we demonstrate that CpG60% is

capable of delivering functional electrical stimulation to support and enhance neurite outgrowth. While the *in vivo* parameters for electrostimulation of different neuronal types have been well explored [59], the effect of stimulation-induced neuronal growth on graphene-based materials is not well documented, although stimulating neural stem cells on ECM-coated graphene supports their differentiation and maturation [47,49]. Therefore, this study advances these findings and demonstrates the ability of the graphene composite to drive strong neurite outgrowth from differentiated adult neurons seeded directly onto the composite surface.

2.4. Application of CpG composites in the creation of neuronal medical devices

With the innate stability, biocompatibility, and responsiveness to electrostimulation of the CpG60% nanocomposite confirmed, we next wanted to assess its versatility for the fabrication of distinct CNS-derived medical device applications. Directionally aligned scaffold micro-architectures are particularly well-suited for supporting anisotropic axonal regrowth for peripheral [101] and central nervous system applications [66]. First, lyophilized nerve guidance scaffolds were produced using directional freeze drying [102] (Fig. 5A), with a highly aligned porous internal architecture visible under SEM (Fig. 5B), and an electrical conductivity of $2 \times 10^{-6} \pm 5 \times 10^{-7}$ S/m. The aligned pore structure is optimally designed to allow cell infiltration and drive longitudinal axonal growth along the neurotrophic conductive collagen matrix, making it an excellent candidate for regenerative medical devices.

Microelectrode arrays are frequently used to record and/or deliver electrical stimulation to discrete neuronal populations, either to restore function, or to connect to a brain-machine interface [103,104]. Therefore, to demonstrate the ability of CpG60% to form fine micron-sized structural features, a custom 5×5 microneedle array, with height 2.5 mm and tip diameter 40–80 μm , was successfully dry cast (Fig. 5C, D), demonstrating the versatility of the composite. Finally, the rapidly evolving field of bioelectronics relies on the fabrication of conductive yet biocompatible circuitry, which can be difficult to achieve because of cytotoxicity issues, the poor processability of conductive polymers or difficulties in printing with traditional conductors [105]. To demonstrate the potential of the CpG60% composite for 3D printing and additive manufacturing applications, it was adapted into a printable bioink, capable of producing repeatable complex electroconductive features (Fig. 5E) with over 5-fold enhanced conductivity (Fig. 5F, ~ 8 S/m c.f. ~ 1.5 S/m for dry cast films) and low spreading ratio (Sup. Fig. 7B). The increase in conductivity is likely due to the decreased alignment of the graphene in the composite, due to the higher viscosity of the printing slurries. Alignment in polymer nanocomposites can give rise to nanosheet junctions with large amounts of polymer between them, increasing the junction resistance, thus decreasing the conductivity [106]. These enhanced properties allow for the printing of functional, high-resolution circuits for bioelectronics, such as the LED circuit shown below, confirming the utility of the material (Fig. 5E, G). Together, these results show that CpG composites are a versatile material which can be readily adapted to a wide variety of relevant bioengineering applications while exhibiting excellent biocompatibility and conductivity. The results also demonstrate the promise of the material for the electrical stimulation of other excitable tissues such as peripheral nerve, muscle and cardiac tissue [14,15,107].

3. Concluding remarks

For next generation neural medical devices, there is an outstanding demand for the development of electroconductive materials that seamlessly integrate with delicate neural tissues, while minimizing deleterious secondary effects such as scarring and reduced bioactivity. In this study we outline the development and characterisation of novel and

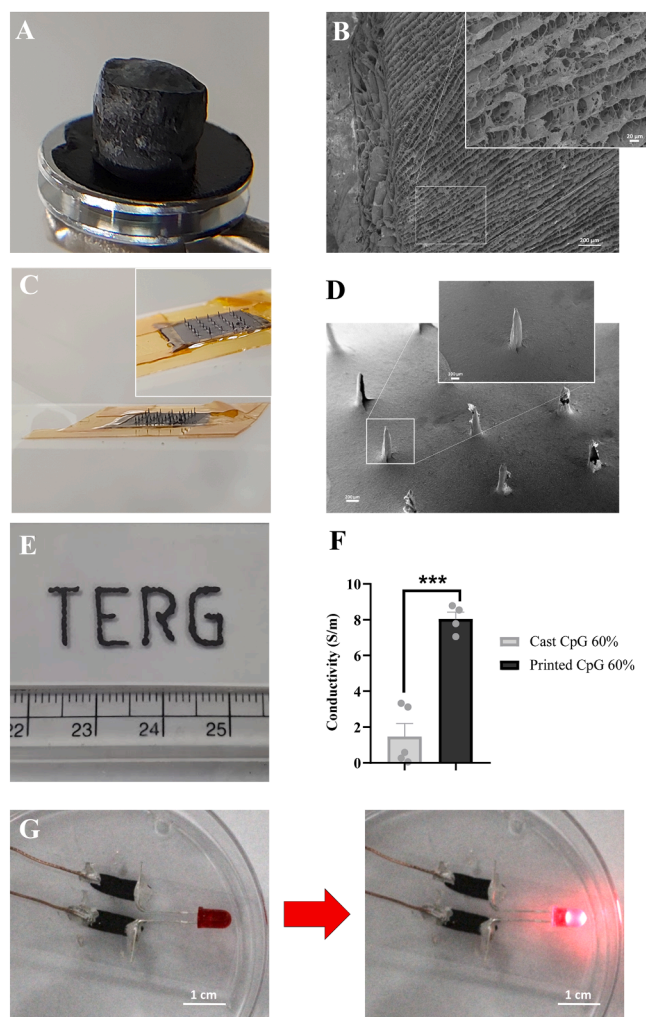


Fig. 5. Applications of conductive collagen/pristine graphene to produce different types of neural interfacing structures. (A,B) CpG60% was used to fabricate lyophilized porous conductive scaffolds (A) that when imaged using SEM (B) show the porous internally aligned channels. Scalebars = 200 μm and 20 μm (inset). (C,D) Microneedle arrays were fabricated using the composite to produce a 5 \times 5 microneedle set (C). Each needle was 2.5 mm high and with a tip diameter of 40–80 μm . (D) when imaged at higher power using SEM (D) the fine bore and sharp tips can be clearly seen on the individual needles. Scalebars = 200 μm and 100 μm (inset). (E,F) The development of the CpG60% as a printable bioink allowed for the control and fine printing of complex 3D geometries (E) with excellent conductivity (F). (G) To demonstrate the conductive function of the CpG60% bioink, a simple circuit was printed to power an LED. Video of circuit functioning available in supplementary material (Sup. Fig. 7A). Scalebars represent 1 cm. *** $p < 0.001$.

versatile type-I collagen/pristine graphene (CpG) nanocomposites that strike the essential balance between physiologically relevant electrical conductivity and neurocompatibility. We identify one formulation, CpG60%, as an ideal candidate capable of supporting and stimulating neuronal growth. Equally significant is its immuno-compatibility, and ability to support the growth and proliferation of CNS astrocytes and microglia with morphologies and cytokine expression profiles typical of non-inflammatory resting phenotypes. When combined with its ability to trophically support iPSC- and primary neuron growth and successfully deliver functional electrical stimulation to enhance the number and length of neurites, these data identify CpG60% as a pan-CNS cell compatible nanocomposite. When coupled with its versatility to produce a range of conductive, neural-interfacing structures, including porous scaffolds, microneedle arrays, and 3D-printed circuits for bioelectronics,

this identifies CpG60% as an excellent candidate nanocomposite for future neuronal medical device applications.

Data availability statement

The data that support the findings of this study are available from the corresponding author, Prof. F.J. O' Brien, upon reasonable request.

CRediT authorship contribution statement

Jack Maughan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Pedro J. Gouveia:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Javier Gutierrez Gonzalez:** Formal analysis, Investigation, Methodology. **Liam M. Leahy:** Investigation, Methodology, Formal analysis. **Ian Woods:** Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Cian O'Connor:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Tara McGuire:** Formal analysis, Investigation, Methodology. **James R. Garcia:** Investigation, Methodology, Writing – review & editing. **Sarah F. McComish:** Investigation, Writing – review & editing. **Oran D. Kennedy:** Resources. **Maeve A. Caldwell:** Resources. **Adrian Dervan:** Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Jonathan N. Coleman:** Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Fergal J. O'Brien:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

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 paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.apmt.2022.101629.

Reference

- [1] A. Marino, G.G. Genchi, V. Mattoli, G. Ciofani, Piezoelectric nanotransducers: the future of neural stimulation, *Nano Today* 14 (2017) 9–12, <https://doi.org/10.1016/j.nantod.2016.12.005>.
- [2] Q. Zheng, Y. Zou, Y. Zhang, Z. Liu, B. Shi, X. Wang, Z.L. Wang, Biodegradable triboelectric nanogenerator as a life-time designed implantable power source, *Sci. Adv.* 2 (3) (2016), e1501478, <https://doi.org/10.1126/sciadv.1501478>.
- [3] G. Conta, A. Libanori, T. Tat, G. Chen, J. Chen, Triboelectric nanogenerators for therapeutic electrical stimulation, *Adv. Mater.* 33 (2021), 2007502, <https://doi.org/10.1002/adma.202007502>.
- [4] D.O. Adewole, M.D. Serruya, J.A. Wolf, D.K. Cullen, Bioactive neuroelectronic interfaces, *Front. Neurosci.* 13 (2019) 269–269.
- [5] F. Lotti, F. Ranieri, G. Vadalà, L. Zollo, G. Di Pino, Invasive intraneural interfaces: foreign body reaction issues, *Front. Neurosci.* 11 (2017) 497–497.
- [6] R. Green, M.R. Abidian, Conducting polymers for neural prosthetic and neural interface applications, *Adv. Mater.* 27 (46) (2015) 7620–7637 (Deerfield Beach, Fla.).
- [7] Y.C. Jeong, H.E. Lee, A. Shin, D.G. Kim, K.J. Lee, D. Kim, Progress in brain-compatible interfaces with soft nanomaterials, *Adv. Mater.* 32 (2020), 1907522.
- [8] Y. Liu, A.F. McGuire, H.Y. Lou, T.L. Li, J.B.H. Tok, B. Cui, Z. Bao, Soft conductive micropillar electrode arrays for biologically relevant electrophysiological recording, *Proc. Natl. Acad. Sci.* 115 (46) (2018) 11718–11723, <https://doi.org/10.1073/pnas.1810827115>.
- [9] G. Lind, C.E. Linsmeier, J. Schouenborg, The density difference between tissue and neural probes is a key factor for glial scarring, *Sci. Rep.* 3 (2013), <https://doi.org/10.1038/SREP02942>.
- [10] K. Kostarelos, M. Vincent, C. Hebert, J.A. Garrido, Graphene in the design and engineering of next-generation neural interfaces, *Adv. Mater.* 29 (42) (2017), 1700909, <https://doi.org/10.1002/adma.201700909>.
- [11] Y. Lu, X. Liu, D. Kuzum, Graphene-based neurotechnologies for advanced neural interfaces, *Curr. Opin. Biomed. Eng.* 6 (2018) 138–147.
- [12] E. Manoussiotakis, J. Park, J.G. Hardy, J.Y. Lee, C.E. Schmidt, Towards the translation of electroconductive organic materials for regeneration of neural tissues, *Acta Biomater.* 139 (2022) 22–42, <https://doi.org/10.1016/j.actbio.2021.07.065>.
- [13] A.R. Spencer, A. Primbetova, A.N. Koppes, R.A. Koppes, H. Fenniri, N. Annabi, Electroconductive gelatin methacryloyl-PEDOT:PSS composite hydrogels: design, synthesis, and properties, *ACS Biomater. Sci. Eng.* 4 (5) (2018) 1558–1567, <https://doi.org/10.1021/acsbomater.8b00135>.
- [14] R. Dong, P.X. Ma, B. Guo, Conductive biomaterials for muscle tissue engineering, *Biomaterials* 229 (2020), 119584, <https://doi.org/10.1016/j.biomaterials.2019.119584>.
- [15] Y. Wu, L. Wang, T. Hu, P.X. Ma, B. Guo, Conductive micropatterned polyurethane films as tissue engineering scaffolds for Schwann cells and PC12 cells, *J. Colloid Interface Sci.* 518 (2018) 252–262, <https://doi.org/10.1016/j.jcis.2018.02.036>.
- [16] Y. Wu, L. Wang, B. Guo, Y. Shao, P.X. Ma, Electroactive biodegradable polyurethane significantly enhanced Schwann cells myelin gene expression and neurotrophin secretion for peripheral nerve tissue engineering, *Biomaterials* 87 (2016) 18–31, <https://doi.org/10.1016/j.biomaterials.2016.02.010>.
- [17] T. Sattar, Current review on synthesis, composites and multifunctional properties of graphene, *Top. Curr. Chem.* 377 (2) (2019) 10.
- [18] A. Xavier Mendes, S. Moraes Silva, C.D. O'Connell, S. Duchi, A.F. Quigley, R.M. I. Kapsa, S.E. Moulton, Enhanced electroactivity, mechanical properties, and printability through the addition of graphene oxide to photo-cross-linkable gelatin methacryloyl hydrogel, *ACS Biomater. Sci. Eng.* 7 (6) (2021) 2279–2295, <https://doi.org/10.1021/acsbomater.0c01734>.
- [19] A. Domínguez-Bajo, A. González-Mayorga, E. López-Dolado, M.C. Serrano, Graphene-derived materials interfacing the spinal cord: outstanding *in vitro* and *in vivo* findings, *Front. Syst. Neurosci.* 11 (2017), <https://doi.org/10.3389/fnsys.2017.00071>.
- [20] S.M. Wellman, J.R. Eles, K.A. Ludwig, J.P. Seymour, N.J. Michelson, W. E. McFadden, A.L. Vazquez, T.D.Y. Kozai, A materials roadmap to functional neural interface design, *Adv. Funct. Mater.* 28 (12) (2018), 1701269.
- [21] R. Rauti, M. Medelin, L. Newman, S. Vranic, G. Reina, A. Bianco, M. Prato, K. Kostarelos, L. Ballerini, Graphene oxide flakes tune excitatory neurotransmission *in vivo* by targeting hippocampal synapses, *Nano Lett.* 19 (5) (2019) 2858–2870, <https://doi.org/10.1021/acs.nanolett.8b04903>.
- [22] J.W. Yang, C.Y. Chen, Z.Y. Yu, J.H.Y. Chung, X. Liu, C.Y. Wu, G.Y. Chen, An electroactive hybrid biointerface for enhancing neuronal differentiation and axonal outgrowth on bio-subretinal chip, *Mater. Today Biol.* 14 (2022), 100253, <https://doi.org/10.1016/j.mtbio.2022.100253>.
- [23] A.K. Geim, Graphene: status and prospects, *Science* 324 (5934) (2009) 1530–1534, <https://doi.org/10.1126/science.1158877>.
- [24] A. Fabbro, D. Scaini, V. León, E. Vázquez, G. Cellot, G. Privitera, L. Lombardi, F. Torrisi, F. Tomarchio, B. Bonaccorso, S. Bosi, A.C. Ferrari, L. Ballerini, M. Prato, Graphene-based interfaces do not alter target nerve cells, *ACS Nano* 10 (1) (2016) 615–623.
- [25] L. Ou, B. Song, H. Liang, et al., Toxicity of graphene-family nanoparticles: a general review of the origins and mechanisms, *Part. Fibre Toxicol.* 13 (2016) 57.
- [26] V.B. Mohan, R. Brown, K. Jayaraman, D. Bhattacharyya, Characterisation of reduced graphene oxide: effects of reduction variables on electrical conductivity, *Mater. Sci. Eng. B* 193 (C) (2015) 49–60, <https://doi.org/10.1016/j.mseb.2014.11.002>.
- [27] Y. Wang, Y. Chen, S.D. Lacey, L. Xu, H. Xie, T. Li, V.A. Danner, L. Hu, Reduced graphene oxide film with record-high conductivity and mobility, *Mater. Today* 21 (2) (2018) 186–192, <https://doi.org/10.1016/j.mattod.2017.10.008>.
- [28] A. Ciesielski, P. Samorì, Graphene via sonication assisted liquid-phase exfoliation, *Chem. Soc. Rev.* 43 (1) (2013) 381–398, <https://doi.org/10.1039/C3CS60217F>.
- [29] E. Sawosz, S. Jaworski, M. Kutwin, A. Hotowy, M. Wierzbiicki, M. Grodzik, N. Kurantowicz, B. Strojny, L. Lipińska, A. Chwalibog, Toxicity of pristine graphene in experiments in a chicken embryo model, *Int. J. Nanomed.* 9 (2014) 3913–3922.
- [30] Y. Li, Y. Liu, Y. Fu, T. Wei, L. Le Guyader, G. Gao, R.S. Liu, Y.Z. Chang, C. Chen, The triggering of apoptosis in macrophages by pristine graphene through the MAPK and TGF-beta signaling pathways, *Biomaterials* 33 (2) (2012) 402–411.
- [31] F. Veliev, A. Briancón-Marjollet, V. Bouchiat, C. Delacour, Impact of crystalline quality on neuronal affinity of pristine graphene, *Biomaterials* 86 (2016) 33–41.
- [32] H.P. Bei, Y. Yang, Q. Zhang, Y. Tian, X. Luo, M. Yang, X. Zhao, Graphene-based nanocomposites for neural tissue engineering, *Molecules* 24 (4) (2019).
- [33] M. Moschetta, J.Y. Lee, J. Rodrigues, A. Podestà, O. Varvicchio, J. Son, Y. Lee, K. Kim, G.H. Lee, F. Benfenati, M. Bramini, A. Capasso, Hydrogenated graphene improves neuronal network maturation and excitatory transmission, *Adv. Biol.* 5 (1) (2021), e2000177.
- [34] M. Gobbi, E. Orgiu, P. Samorì, When 2D materials meet molecules: opportunities and challenges of hybrid organic/inorganic van der waals heterostructures, *Adv. Mater.* 30 (18) (2018), 1706103.
- [35] Y. Hernandez, V. Nicolosi, M. Lotya, F.M. Blighe, Z. Sun, S. De, I.T. McGovern, B. Holland, M. Byrne, Y.K. Gun'ko, J.J. Boland, P. Niraj, G. Duesberg, S. Krishnamurthy, R. Goodhue, J. Hutchison, V. Scardaci, A.C. Ferrari, J. N. Coleman, High-yield production of graphene by liquid-phase exfoliation of graphite, *Nat. Nanotechnol.* 3 (9) (2008) 563–568.
- [36] A. Bendali, L.H. Hess, M. Seifert, V. Forster, A.-F. Stephan, J.A. Garrido, S. Picaud, Purified neurons can survive on peptide-free graphene layers, *Adv. Healthc. Mater.* 2 (2013) 929–933, <https://doi.org/10.1002/adhm.201200347>.
- [37] F. Lebre, J.B. Boland, P. Gouveia, A.L. Gorman, M.L.E. Lundahl, R.I. Lynch, F. J. O'Brien, J. Coleman, E.C. Lavelle, Pristine graphene induces innate immune training, *Nanoscale* (2020).
- [38] A.J. Ryan, C.J. Kearney, N. Shen, U. Khan, A.G. Kelly, C. Probst, E. Draehle, S. Biccari, C.D. Garcarena, V. Vega-Mayoral, P. Loskill, S.W. Kerrigan, D.J. Kelly, K. Schenke-Layland, J.N. Coleman, F.J. O'Brien, Electroconductive biohybrid collagen/pristine graphene composite biomaterials with enhanced biological activity, *Adv. Mater.* 30 (15) (2018), 1706442.
- [39] P. May, U. Khan, A. O'Neill, J.N. Coleman, Approaching the theoretical limit for reinforcing polymers with graphene, *J. Mater. Chem.* 22 (2012) 1278–1282, <https://doi.org/10.1039/C1JM15467B>.
- [40] Y. Shin, X. Just-Baringo, M. Boyes, A. Panigrahi, M. Zarattini, Y. Chen, X. Liu, G. Morris, E. Prestat, K. Kostarelos, S. Vranic, Igor Larrosa and Cinzia Casiraghi, Enhanced liquid phase exfoliation of graphene in water using an insoluble bis-pyrene stabiliser, *Faraday Discuss.* 227 (2021) 46–60.
- [41] S.K. Rastogi, G. Raghavan, G. Yang, T. Cohen-Karni, Effect of graphene on nonneuronal and neuronal cell viability and stress, *Nano Lett.* 17 (5) (2017) 3297–3301, https://doi.org/10.1021/acs.nanolett.7b01215/SUPPL_FILE/NL7B01215_SI_001.PDF.
- [42] G. Agarwal, N. Kumar, A. Srivastava, Highly elastic, electroconductive, immunomodulatory graphene crosslinked collagen cryogel for spinal cord regeneration, *Mater. Sci. Eng. C* 118 (2021), <https://doi.org/10.1016/j.msec.2020.111518>.
- [43] S. Pok, F. Vitale, S.L. Eichmann, O.M. Benavides, M. Pasquali, J.G. Jacot, Biocompatible carbon nanotube-chitosan scaffold matching the electrical conductivity of the heart, *ACS Nano* 8 (10) (2014) 9822–9832, <https://doi.org/10.1021/nm503693h>.
- [44] M. Uz, M. Donta, M. Mededovic, D.S. Sakaguchi, S.K. Mallapragada, Development of gelatin and graphene-based nerve regeneration conduits using three-dimensional (3D) printing strategies for electrical transdifferentiation of mesenchymal stem cells, *Ind. Eng. Chem. Res.* 58 (18) (2019) 7421–7427, <https://doi.org/10.1021/acs.iecr.8b05537>.
- [45] K. Kostarelos, K.S. Novoselov, Materials science. Exploring the interface of graphene and biology, *Science* 344 (6181) (2014) 261–263.
- [46] N. Li, X. Zhang, Q. Song, R. Su, Q. Zhang, T. Kong, L. Liu, G. Jin, M. Tang, G. Cheng, The promotion of neurite sprouting and outgrowth of mouse hippocampal cells in culture by graphene substrates, *Biomaterials* 32 (35) (2011 Dec) 9374–9382, <https://doi.org/10.1016/j.biomaterials.2011.08.065>.
- [47] S.Y. Park, J. Park, S.H. Sim, M.G. Sung, K.S. Kim, B.H. Hong, S. Hong, Enhanced differentiation of human neural stem cells into neurons on graphene, *Adv. Mater.* 23 (36) (2011) H263–H267, <https://doi.org/10.1002/adma.201101503>.
- [48] Z. He, S. Zhang, Q. Song, W. Li, D. Liu, H. Li, M. Tang, R. Chai, The structural development of primary cultured hippocampal neurons on a graphene substrate, *Colloids Surf. B Biointerfaces* 146 (2016) 442–451, <https://doi.org/10.1016/j.colsurfb.2016.06.045>.
- [49] M. Tang, Q. Song, N. Li, Z. Jiang, R. Huang, G. Cheng, Enhancement of electrical signalling in neural networks on graphene films, *Biomaterials* 34 (27) (2013) 6402–6411, <https://doi.org/10.1016/j.biomaterials.2013.05.024>. SepEpub 2013 Jun 4. PMID: 23755830.
- [50] G. Lind, C.E. Linsmeier, J. Thelin, J. Schouenborg, Gelatine-embedded electrodes - a novel biocompatible vehicle allowing implantation of highly flexible microelectrodes, *J. Neural Eng.* 7 (4) (2010), <https://doi.org/10.1088/1741-2560/7/4/046005>.
- [51] W. Shen, L. Karumbaiah, X. Liu, T. Saxena, S. Chen, R. Patkar, R.v. Bellamkonda, M.G. Allen, Extracellular matrix-based intracortical microelectrodes: Toward a

- microfabricated neural interface based on natural materials, *Microsyst. Nanoeng.* 1 (2015), <https://doi.org/10.1038/micronano.2015.10>.
- [52] F. Vitale, W. Shen, N. Driscoll, J.C. Burrell, A.G. Richardson, O. Adewole, B. Murphy, A. Ananthakrishnan, H. Oh, T. Wang, T.H. Lucas, D. Kacy Cullen, M. G. Allen, B. Litt, Biomimetic extracellular matrix coatings improve the chronic biocompatibility of microfabricated subdural microelectrode arrays, *PLoS One* 13 (11) (2018), <https://doi.org/10.1371/journal.pone.0206137>.
- [53] L. Koessler, S. Colnat-Coulbois, T. Cecchin, J. Hofmanis, J.P. Dmochowski, A. M. Norcia, L.G. Maillard, *In-vivo* measurements of human brain tissue conductivity using focal electrical current injection through intracerebral multicontact electrodes, *Hum. Brain Mapp.* 38 (2) (2017) 974–986.
- [54] R. Boni, A. Ali, A. Shavandi, et al., Current and novel polymeric biomaterials for neural tissue engineering, *J. Biomed. Sci.* 25 (2018) 90.
- [55] C. Dong, Y. Lv, Application of collagen scaffold in tissue engineering: recent advances and new perspectives, *Polymers* 8 (2) (2016) 42.
- [56] L.R. Doblado, C. Martínez-Ramos, M.M. Pradas, Biomaterials for neural tissue engineering, *Front. Nanotechnol.* 3 (2021) 21, pp26 April.
- [57] F. Lebre, D. Hanlon, J.B. Boland, J. Coleman, E.C. Lavelle, Exfoliation in endotoxin-free albumin generates pristine graphene with reduced inflammatory properties, *Adv. Biosyst.* 2 (12) (2018), <https://doi.org/10.1002/adbi.201800102>.
- [58] J. Huang, L. Lu, J. Zhang, X. Hu, Y. Zhang, W. Liang, S. Wu, Z. Luo, Electrical stimulation to conductive scaffold promotes axonal regeneration and remyelination in a rat model of large nerve defect, *PLoS One* 7 (6) (2012) e39526.
- [59] C. Bertucci, R. Koppes, C. Dumont, Abigail Koppes, Neural responses to electrical stimulation in 2D and 3D *in vitro* environments, *Brain Res. Bull.* 152 (2019) 265–284.
- [60] D.G. Papageorgiou, I.A. Kinloch, R.J. Young, Graphene/elastomer nanocomposites, *Carbon* 460–484 (2015) 95.
- [61] R.J. Young, M. Liu, I.A. Kinloch, S. Li, X. Zhao, C. Vallés, D.G. Papageorgiou, The mechanics of reinforcement of polymers by graphene nanoplatelets, *Compos. Sci. Technol.* 154 (2018) 110–116, <https://doi.org/10.1016/j.compscitech.2017.11.007>.
- [62] P. Fratzl, K. Misof, I. Zizak, G. Rapp, H. Amenitsch, S. Bernstorff, Fibrillar structure and mechanical properties of collagen, *J. Struct. Biol.* 122 (1–2) (1998) 119–122, <https://doi.org/10.1006/jsbi.1998.3966>. IssuesPagesISSN 1047-8477.
- [63] R. Akhtar, M.J. Sherratt, J.K. Cruickshank, B. Derby, Characterizing the elastic properties of tissues, *Mater. Today* 14 (3) (2011) 96–105, [https://doi.org/10.1016/S1369-7021\(11\)70059-1](https://doi.org/10.1016/S1369-7021(11)70059-1). ISSN 1369-7021.
- [64] T.D.Y. Kozai, K. Catt, X. Li, Z.V. Gugel, V.T. Olafsson, A.L. Vazquez, X.T. Cui, Mechanical failure modes of chronically implanted planar silicon-based neural probes for laminar recording, *Biomaterials* 37 (2015) 25.
- [65] A. Kourgiantaki, D.S. Tzeranis, K. Karali, K. Georgelou, E. Bampoula, S. Psilodimitrakopoulos, I.v. Yannas, E. Stratakis, K. Sidiropoulou, I. Charalampopoulos, A. Gravanis, Neural stem cell delivery via porous collagen scaffolds promotes neuronal differentiation and locomotion recovery in spinal cord injury, *NPJ Regen. Med.* 5 (1) (2020) 1–14, <https://doi.org/10.1038/s41536-020-0097-0>, 2020 5:1.
- [66] I. Woods, C. O'Connor, L. Frugoli, S. Kerr, J. Gutierrez, M. Stasiewicz, T. McGuire, B. Cavanagh, A. Hibbitts, A. Dervan, F.J. O'Brien, Biomimetic scaffolds for spinal cord applications exhibit stiffness-dependent immunomodulatory and neurotrophic characteristics, *Adv. Healthc. Mater.* 11 (2022), 2101663, <https://doi.org/10.1002/adhm.202101663>.
- [67] B. Ch. Kholkhoev, A.S. Buinov, V.G. Makotchenko, V.E. Fedorov, P.S. Timashev, V.F. Burdukovskii, Electrically conductive composites of collagen and graphene, *Russ. Chem. Bull. Int. Ed.* 67 (7) (2018) 1316–1318.
- [68] S. Cai, C. Wu, W. Yang, W. Liang, H. Yu, L. Liu, Recent advance in surface modification for regulating cell adhesion and behaviors, *Nanotechnol. Rev.* 9 (1) (2020) 971–989, <https://doi.org/10.1515/ntrv-2020-0076>.
- [69] E. Biazar, M. Heidari, A. Asefnejad, N. Montazeri, The relationship between cellular adhesion and surface roughness in polystyrene modified by microwave plasma radiation, *Int. J. Nanomed.* 6 (2011) 631–639, <https://doi.org/10.2147/IJN.S17218>.
- [70] A. Zareidoost, M. Yousefpour, B. Ghaseme, A. Amanzadeh, The relationship of surface roughness and cell response of chemical surface modification of titanium, *J. Mater. Sci. Mater. Med.* 23 (6) (2012) 1479–1488, <https://doi.org/10.1007/s10856-012-4611-9>.
- [71] V. Brunetti, G. Maiorano, L. Rizzello, B. Sorce, S. Sabella, R. Cingolani, P.P. Pompa, Neurons sense nanoscale roughness with nanometer sensitivity, *Proc. Natl. Acad. Sci. U. S. A.* 107 (14) (2010) 6264–6269, <https://doi.org/10.1073/pnas.0914456107>.
- [72] D. Sahni, A. Jea, J.A. Mata, D.C. Marcano, A. Sivaganesan, J.M. Berlin, C. E. Tatsui, Z. Sun, T.G. Luerssen, S. Meng, T.A. Kent, J.M. Tour, Biocompatibility of pristine graphene for neuronal interface, *J. Neurosurg. Pediatr.* 11 (5) (2013) 575–583, <https://doi.org/10.3171/2013.1.PEDS12374>.
- [73] F. Gentile, L. Tirinato, E. Battista, F. Causa, C. Liberale, E.M. di Fabrizio, P. Decuzzi, Cells preferentially grow on rough substrates, *Biomaterials* 31 (28) (2010) 7205–7212, <https://doi.org/10.1016/j.biomaterials.2010.06.016>.
- [74] A. Carnicer-Lombarte, C. ST, G.G. Malliaras, DG. Barone, Foreign body reaction to implanted biomaterials and its impact in nerve neuroprosthetics, *Front. Bioeng. Biotechnol.* 9 (2021), 622524, <https://doi.org/10.3389/fbioe.2021.622524>.
- [75] E.J. Bradbury, E.R. Burnside, Moving beyond the glial scar for spinal cord repair, *Nat. Commun.* 10 (2019) 3879, <https://doi.org/10.1038/s41467-019-11707-7>.
- [76] M.M. Halassa, P.G. Haydon, Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior, *Ann. Rev. Physiol.* 72 (2010) 335–355, <https://doi.org/10.1146/annurev-physiol-021909-135843>.
- [77] Z. Gao, Q. Zhu, Y. Zhang, Y. Zhao, L. Cai, C.B. Shields, J. Cai, Reciprocal modulation between microglia and astrocyte in reactive gliosis following the CNS injury, *Mol. Neurobiol.* 48 (3) (2013) 690–701, <https://doi.org/10.1007/s12035-013-8460-4>.
- [78] V. Bellver-Landete, F. Bretheau, B. Mailhot, N. Vallières, M. Lessard, M.E. Janelle, N. Vernoux, M.E. Tremblay, T. Fuehrmann, M.S. Shoichet, S. Lacroix, Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury, *Nat. Commun.* 10 (1) (2019) 518, <https://doi.org/10.1038/s41467-019-08446-0>.
- [79] R. Sridharan, B. Cavanagh, A.R. Cameron, D.J. Kelly, F.J. O'Brien, Material stiffness influences the polarization state, function and migration mode of macrophages, *Acta Biomater.* 89 (2019) 47–59, <https://doi.org/10.1016/j.actbio.2019.02.048>.
- [80] M.C.P. Mendonça, E.S. Soares, M.B. de Jesus, et al., Reduced graphene oxide: nanotoxicological profile in rats, *J. Nanobiotechnol.* 14 (2016) 53, <https://doi.org/10.1186/s12951-016-0206-9>.
- [81] M. Durso, A.I. Borrachero-Conejo, C. Bettini, E. Treossi, A. Scidà, E. Saracino, M. Gazzano, M. Christian, V. Morandi, G. Tuci, G. Giambastiani, L. Ottaviano, F. Perrozzi, V. Benfenati, M. Melucci, V. Palermo, Biomimetic graphene for enhanced interaction with the external membrane of astrocytes, *J. Mater. Chem. B* 6 (33) (2018) 5335–5342, <https://doi.org/10.1039/c8tb01410h>.
- [82] M. Bramini, M. Chiacchiaretta, A. Armirotti, A. Rocchi, D.D. Kale, C. Martin, E. Vázquez, T. Bandiera, S. Ferroni, F. Cesca, F. Benfenati, An increase in membrane cholesterol by graphene oxide disrupts calcium homeostasis in primary astrocytes, *Small* 15 (2019), 1900147, <https://doi.org/10.1002/sml.201900147>.
- [83] R. Fabbri, E. Saracino, E. Treossi, R. Zamboni, V. Palermo, V. Benfenati, Graphene glial-interfaces: challenges and perspectives, *Nanoscale* 13 (8) (2021) 4390–4407, <https://doi.org/10.1039/d0nr07824g>.
- [84] M. Chiacchiaretta, M. Bramini, A. Rocchi, A. Armirotti, E. Giordano, E. Vázquez, T. Bandiera, S. Ferroni, F. Cesca, F. Benfenati, Graphene oxide upregulates the homeostatic functions of primary astrocytes and modulates astrocyte-to-neuron communication, *Nano Lett.* 18 (9) (2018) 5827–5838, <https://doi.org/10.1021/acs.nanolett.8b02487>.
- [85] M.K. Gottipati, I. Kalinina, E. Bekyarova, R.C. Haddon, V. Parpura, Chemically functionalized water-soluble single-walled carbon nanotubes modulate morphological characteristics of astrocytes, *Nano Lett.* 12 (9) (2012) 4742–4747, <https://doi.org/10.1021/nl302178s>.
- [86] A. Matejuk, R.M. Ranshoff, Crosstalk between astrocytes and microglia: an overview, *Front. Immunol.* 11 (2020) 1416, <https://doi.org/10.3389/fimmu.2020.01416>.
- [87] Z. Gao, Q. Zhu, Y. Zhang, Y. Zhao, L. Cai, C.B. Shields, et al., Reciprocal modulation between microglia and astrocyte in reactive gliosis following the CNS injury, *Mol. Neurobiol.* 48 (2013) 690–701, <https://doi.org/10.1007/s12035-013-8460-4>.
- [88] S.A. Elw, B.A. Barres, Reactive astrocytes: production, function, and therapeutic potential, *Immunity*. 46 (6) (2017) 957–967, <https://doi.org/10.1016/j.immuni.2017.06.006>.
- [89] Y. Tang, W. Le, Differential roles of M1 and M2 microglia in neurodegenerative diseases, *Mol. Neurobiol.* 53 (2) (2016) 1181–1194, <https://doi.org/10.1007/s12035-014-9070-5>.
- [90] K. Zhou, S. Motamed, G.A. Thouas, C.C. Bernard, D. Li, H.C. Parkington, H. A. Coleman, D.I. Finkelstein, J.S. Forsythe, Graphene functionalized scaffolds reduce the inflammatory response and supports endogenous neuroblast migration when implanted in the adult brain, *PLoS One* 11 (3) (2016), e0151589.
- [91] Q. Song, Z. Jiang, N. Li, P. Liu, L. Liu, M. Tang, G. Cheng, Anti-inflammatory effects of three-dimensional graphene foams cultured with microglial cells, *Biomaterials* 35 (25) (2014) 6930–6940, <https://doi.org/10.1016/j.biomaterials.2014.05.002>.
- [92] Z. Yang, Y. Pan, T. Chen, L. Li, W. Zou, D. Liu, D. Xue, X. Wang, G. Lin, Cytotoxicity and immune dysfunction of dendritic cells caused by graphene oxide, *Front. Pharmacol.* 11 (2020) 1206, <https://doi.org/10.3389/fphar.2020.01206>.
- [93] A. Ledebuer, J.J.P. Brevé, A. Wierinckx, S. Van Der Jagt, A.F. Bristow, J.E. Leyens, F.J.H. Tilders, A.M. Van Dam, Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells, *Eur. J. Neurosci.* 16 (2002) 1175–1185, <https://doi.org/10.1046/j.1460-9568.2002.02200.x>.
- [94] K.M. Szostak, L. Grand, T.G. Constantinou, Neural interfaces for intracortical recording: requirements, fabrication methods, and characteristics, *Front. Neurosci.* 11 (2017) 665, <https://doi.org/10.3389/fnins.2017.00665>.
- [95] M. Ferguson, D. Sharma, D. Ross, F. Zhao, A critical review of microelectrode arrays and strategies for improving neural interfaces, *Adv. Healthc. Mater.* 8 (19) (2019), 1900558, <https://doi.org/10.1002/ADHM.201900558>.
- [96] H. Cui, X. Xie, S. Xu, L.L.H. Chan, Y. Hu, Electrochemical characteristics of microelectrode designed for electrical stimulation, *BioMed. Eng. Online* 18 (1) (2019) 1–14, <https://doi.org/10.1186/s12938-019-0704-8>.
- [97] Q. Zhang, D. Esrafilzadeh, J.M. Crook, R. Kapsa, E.M. Stewart, E. Tomaskovic-Crook, G.G. Wallace, Xu Feng Huang, Electrical stimulation using conductive polymer polypyrrole counters reduced neurite outgrowth of primary prefrontal cortical neurons from NRG1-KO and DISC1-LI mice, *Sci. Rep.* 7 (2017) 1–8.
- [98] J.M. Pemberton, J.P. Pogmore, D.W. Andrews, Neuronal cell life, death, and axonal degeneration as regulated by the BCL-2 family proteins, *Cell Death Differ.* 28 (2021) 108–122, <https://doi.org/10.1038/s41418-020-00654-2>.
- [99] M.F. Najafi, S. Zahri, F. Vahedi, L.E. Toosi, N. Ariaee, Which form of collagen is suitable for nerve cell culture? *Neural Regen. Res.* 8 (23) (2013) 2165, <https://doi.org/10.3969/J. ISSN.1673-5374.2013.23.006>.

- [100] W.S. Deng, K. Ma, B. Liang, X.Y. Liu, H.Y. Xu, J. Zhang, H.Y. Shi, H.T. Sun, X. Y. Chen, S. Zhang, Collagen scaffold combined with human umbilical cord-mesenchymal stem cells transplantation for acute complete spinal cord injury, *Neural Regen. Res.* 15 (9) (2020) 1686–1700, <https://doi.org/10.4103/1673-5374.276340>.
- [101] M. Farokhi, F. Mottaghtalab, M.R. Saeb, S. Shojaei, N.K. Zarrin, S. Thomas, S. Ramakrishna, Conductive biomaterials as substrates for neural stem cells differentiation towards neuronal lineage cells, *Macromol. Biosci.* 21 (2021), 2000123.
- [102] WA. Lackington, AJ. Ryan, FJ. O'Brien, Advances in nerve guidance conduit-based therapeutics for peripheral nerve repair, *ACS Biomater. Sci. Eng.* 3 (7) (2017) 1221–1235, <https://doi.org/10.1021/acsbomaterials.6b00500>.
- [103] Z. Xiang, J. Liu, C. Lee, A flexible three-dimensional electrode mesh: an enabling technology for wireless brain-computer interface prostheses, *Microsyst. Nanoeng.* 2 (2016) 16012.
- [104] D. Yan, et al., Microneedle penetrating array with axon-sized dimensions for cuff-less peripheral nerve interfacing, in: *Proceedings of the 9th International IEEE/EMBS Conference on Neural Engineering (NER)*, 2019, pp. 827–830.
- [105] L.E.A Murr, Metallographic review of 3D printing/additive manufacturing of metal and alloy products and components, *Metallogr. Microstruct. Anal.* 7 (2018) 103–132, <https://doi.org/10.1007/s13632-018-0433-6>.
- [106] AG. Kelly, et al., The electrical conductivity of solution-processed nanosheet networks, *Nat. Rev. Mater.* 2021 (2021) 1–18.
- [107] K. Ashtari, H. Nazari, H. Ko, P. Tebon, M. Akhshik, M. Akbari, S.N. Alhosseini, M. Mozafari, B. Mehravi, M. Soleimani, R. Ardehali, M.E. Warkiani, S. Ahadian, A. Khademhosseini, Electrically conductive nanomaterials for cardiac tissue engineering, *Adv. Drug. Deliv. Rev.* 144 (2019) 162–179, <https://doi.org/10.1016/j.addr.2019.06.001>. ISSN 0169-409X.