Fluorinated Graphene for Promoting Neuro-Induction of Stem Cells

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Stem cells play a pivotal role in the human body by providing a repair system for organs and tissues needed for their continual growth and renewal throughout ontogeny. Stem cells are now used as an integral part of modern clinical treatment.[1] Intensive research has been carried out on stem cells to understand the complex molecular and cellular events occurring during early development, disease progression, epigenetics, and pathophysiology.[2] Bone marrow derived mesenchymal stem cells (MSCs) have the potential to differentiate into tissues of mesenchymal origin including adipocytes, osteoblasts and chondrocytes.[3] Whilst MSCs were initially believed to be restricted to the mesenchymal lineages, recent evidence suggests that MSCs can trans-differentiate into tissues of non-mesenchymal origin such as the neurons. This has generated great interests about the potential use of MSCs for the treatment of degenerative and autoimmune diseases of the nervous system.[4,5] However, the mechanisms and the process of trans-differentiation of MSCs to neurons are still not well understood and the occurrence of full functional neural differentiation is still debatable.[6]

Surface chemistry plays an important role in terms of the adhesion properties of proteins and cells. These are mediated at the molecular level by specific functional groups like amine, carboxylic acid and hydroxyl groups. Surfaces with good biocompatibility and tissue-specific inductive capabilities are highly desirable in various tissue engineering and cell therapeutic applications. Previous work has shown that graphene and graphene oxide (GO)[7] can influence cell behaviour, including attachment, growth, proliferation and differentiation, due in part to its aromatic scaffold which can increase the local concentration of extracellular matrix (ECM) such as collagen, laminin and fibronectin by non-covalent binding. It is interesting to consider functionalized graphene such as fluorinated graphene,[8] which is distinct from its polymeric analog such as PTFE due to its highly directional and polar bonding on the surface. The strong polarity of the carbon-fluorine bond is expected to induce biological responses.[9] For example, the introduction of fluorine into pharmaceutical drugs improves metabolic stability and increase the strength of interaction with targeted proteins.[10] The presence of fluorne affects the properties of molecules due to its high electronegativity and small size. Thus, approximately 30% of all agrochemicals and 20% of all pharmaceuticals drugs contain fluoride,[10] including drugs such as Lipitor, Lexapro and Prozac.

In this work, we used fluorinated graphene sheets as the scaffold for stem cell growth. Changes in cellular adhesion, morphology, gene expression and differentiation have been rationalised in terms of the surface chemistry, topography and mechanical properties of the substrate on which the cells have been cultured. Our results showed that fluorinated graphene (FG) induced higher proliferation and stronger polarization of MSCs. Morphological changes in terms of cytoskeletal and nuclear alignment promote the differentiation of MSCs towards the neuronal lineage and the effect can be further enhanced with the addition of neuron-inductive agent, retinoic acid. More importantly, by leveraging on alignment induced neuronal differentiation, the stem cells can be controllably patterned on fluorinated graphene, pointing to a large-scale and rapid strategy to align MSCs and the ability to induce neuronal lineage in the absence of chemical inducer.

The fluorination of graphene was performed by exposing the samples to a fluorinating agent, Xenon difluoride (XeF₂, from Alfa Aesar). The coverage of fluorine on the graphene sample can be controlled by the exposure time in XeF₂ atmosphere. Raman spectroscopy, X-ray Photoelectron Spectroscopy (XPS) and High Resolution Electron Energy Loss Spectroscopy (HREELS) were carried out to evaluate the bonding states of carbon atoms in graphene following the fluorination. The Raman spectra (514 nm) collected for pristine and fluorinated graphene on SiO₂/Si substrates for different length of fluorination times are shown in Figure 1a. The pristine graphene shows the characteristic G (~1587 cm⁻¹) and 2D (~2688 cm⁻¹) band. With increasing fluorination time, a D-band induced by defects or sp³ bonding emerges at 1350 cm⁻¹, while a new peak (~1618 cm⁻¹) splits off from the G band (called D’ peak) and the intensity of the 2D band decreases. These new features are analogous to the formation of sp³ graphane phases and are well documented.[11] When the exposure time is extended to 3600s, the characteristic Raman peaks of pristine graphene completely disappear. This indicates that the aromatic π-electron conjugation...
is totally destroyed, due to the double-sided functionalization of the graphene sheet.[8] At this point, a characteristic ratio of 50% F/C peak intensity in the XPS spectra is designated as double-side fluorinated graphene.[12] UV-vis spectroscopy was used also to probe for the effect of fluorination on π-bonded electronic configuration of graphene. The spectrum of pristine graphene shows a single broad absorption peak at 270 nm which is characteristic of π-π* electron transition in the aromatic system of graphene film. After fluorination, a blue shift (~43 nm) is found because of the polarization-induced charge effect.[13] The XPS C1s and F1s spectra of the fluorinated graphene are shown in Figure 1c and 1d, respectively. The carbon peak is observed at 284.6 eV for the pristine graphene sample, while a chemically shifted component at ~287.5 eV can be assigned to the C-F binding state by comparison with literature data on fluorinated graphite.[14] From the F 1s spectra, we find the C-F bond type changes from ‘semi-ionic’ (685.5 eV binding energy) to covalent (687.5 eV binding energy) with increasing fluorination.
differences in cell morphology and density. The actin cytoskeletal filaments were stained with phalloidin and observed under an epi-fluorescence microscope. As shown in Figure 2c, MSCs cultured on FG proliferate faster and reach high confluence on day 7 as compared to those cultured on PFG and G. The full fluorination of graphene achieves a nearly three-fold increase in cell density, showing that the introduction of C-F bonding on the surface of graphene facilitates better cell adhesion and proliferation (Figure 2d). MSCs on FG are more spindly and elongated which can be attributed to the high density and close packing of the cells. The F-actin fibers are mostly stretched along the long axis of the cells. In contrast, MSCs cultured on PFG and G are less elongated and more widespread.

Previous studies have shown that hydrophilic graphene oxide (GO) can bind to serum proteins via electrostatic interactions, which contributes to a higher density of adhesion molecules available for cell attachment and growth.\[16\] In the case of C-F bonds, it is expected that electrostatic interactions play an important role in determining the biological response to surfaces.\[17\] To investigate the relationship between the surface of fluorinated graphene and the controlled growth of MSCs on
it, the morphology and wettability of surfaces were measured by atomic force microscopy (AFM) and water contact angle (WCA), respectively. With increasing surface coverage of fluorine, the root mean square (RMS) roughness increases from 0.75 nm (graphene) to 3.16 nm (fully fluorinated graphene). After full fluorination, the water contact angle of graphene surface reduces from 83° to ~1° (The insets in Figure 2e–g). The lower contact angle of water is caused by a decrease in the free surface energy of the solid/liquid interface (γSL) resulting from hydrogen bonding.[18] In Figure 2j, the surface free energy of samples is calculated by the Owens-Wendt model: γSL = (1+cosθ) γL, where γSL is the solid-liquid interfacial energy; θ is the contact angle; the surface tension of deionized water (γL, mN m⁻¹) at 20 °C is 72.8 from Lamour et al.[19] The conventional hydrophobicity of fluorinated surfaces is due to the inefficient packing of fluorocarbons on surfaces, leading to poor hydrogen bonding or long range dipole-dipole interactions with water molecules.[20] Theoretical calculation and experiments demonstrated that fluorine coverage saturates at C₄F for one-sided full fluorination, which corresponds to one fluorine atom every two primitive graphene unit cells.[26] Such high coverage of fluorine atoms on a single sheet of carbon present an ordered array of oriented dipoles that can interact with the molecular dipoles of water. Meanwhile, H-bonding interactions also contribute to the decrease of the measured water contact angle. Although the fluorine lone pairs are in conjugation with the local π-orbital system of graphene, C-F···H-O interaction may result from a balance of hyperconjugative bond weakening and rehybridization promoted bond strengthening (Figure 2h).[21] This is in good agreement with the increased wetting properties of fluorinated PDMS surface treated by low pressure CF₄ plasma.[22]

Recent works have demonstrated that MSC lineage specification and cell maturation can be directed by various physical stimuli such as substrate stiffness and cell shape.[23,24] The mechanical tension induces changes in the dynamic and organization of many intra-cellular structures, thereby resulting in changes in gene and protein expression and the phenotype of MSCs. Since cytoskeleton and nucleus elongation is known to induce neuronal differentiation,[25] the elongation index (E) of the nucleus of the cells plated on FG and G is measured using tissue culture polystyrene (PS) as control (See Supporting Information). Based on the statistical results, the nucleus elongation of MSCs cultured on FG is significantly higher than those cultured on G and PS (p < 0.05).

The observed differences could be attributed to the polarization effect of the carbon-fluorine bond which may facilitate cell alignment and nucleus elongation through electrostatic induction at the interface of cell-FG. In addition to morphological changes, neuronal markers- Tuj1 and MAP2, are highly expressed in MSCs cultured on FG as shown by the immunofluorescence staining (Figure 3a). However, co-expression of Nestin is weak in all samples (Figure 3b). The glial specific marker-Glia-fibrillary acidic protein (GFAP) is weakly detected, suggesting a preference of morphological induced differentiation towards the neuronal lineage over the glial lineage. We note that at day 21, compared with the MSCs cultured on G and PS, MSCs cultured on FG demonstrated neuron-like morphologies with visible neurite protrusions indicating further neuronal maturation (see Supporting Information).

Neuronal differentiation of MSCs has been extensively studied and garnered wide interest in the fields of tissue engineering and cell therapy due to their ready availability from the donor bone marrow.[26] To further enhance neuronal differentiation of MSCs, we examined the expression of neuronal gene markers in the neuronal induction culture with retinoic acid. The expression of neuronal markers- Tuj1 and MAP2 was further enhanced when the MSCs were cultured in the presence of 30 μM of retinoic acid (Figure 3b and 3e). The expression of GFAP was slightly enhanced for the MSCs cultured on FG (Figure 3c). Our results show that FG promotes the

Figure 3. Fluorescent images of immunostained MSCs for (a) Tuj1 and GFAP and (d) Nestin and MAP2 cultured on FG, G and PS in the presence of retinoic acid (+RA) and in the absence of retinoic acid (-RA). (a) Tuj1 is shown in red, GFAP in green and (d) Nestin is shown in red, MAP2 is shown in green. Cell nucleus is counter-stained with DAPI in blue (scale bar = 100 μm). Representative areas of the samples were taken. Percentage of immuno-reactive MSCs for (b) Tuj1 (c) GFAP, (e) MAP2 and (f) Nestin on FG, G and PS. Note that more MSCs expressed Tuj1 and MAP2 when cultured on FG and the expression level is further enhanced with the addition of retinoic acid. (n = 6, *p < 0.05).
neuronal differentiation of MSCs and the effect could be further enhanced with the addition of neuron-inductive agent.

In addition to chemical effect, biomechanical signals can also be transmitted to cells via various substrate topographical features such as pillars, grooves or pits. The physical or mechanical stimuli alone is increasingly considered to be sufficient in specifying stem cell lineages which might alleviate the need for chemical factor stimulation. Since distinct morphological characteristics often accompany stem cell differentiation, we investigated whether enhanced neural differentiation can be induced by confining the MSCs into microchannels of FG in the absence of chemical inducers. In Figure 4a, polydimethylsiloxane (PDMS diluted in hexane was utilized as ink) lines were ink-jet printed onto the fluorinated graphene substrates, using a GIX Microplotter II inkjet printer. A typical optical microscopic image of the printed PDMS lines is shown in Figure 4b. The printed PDMS lines have lengths of 3 mm and widths of 150 μm with a line spacing of ∼50 μm resulting in a 30 μm wide FG microchannel in between the PDMS lines. It can be seen in Figure 4c to 4e that the density of aligned MSCs correlates with the coverage of fluorine on the graphene film. Interestingly, randomly seeded MSCs preferentially attach onto the FG microchannels and assume a more elongated morphology. MSCs were cultured on the micro-patterns for 14 days and the expression of neuronal markers analyzed. The morphology of MSCs was examined by F-actin staining. As shown in Figure 4f and Figure 4g, MSCs preferentially adhere onto the FG strips and their cytoskeleton aligns along the length of the pattern. Compared with the cells cultured under non-patterned FG, those cultured in 30 μm FG microchannels demonstrate enhanced expression of Tuj1 and MAP2 even in the absence of neuron-inductive agent (Figure 4h). We note that our finding is consistent with the work by Leong and co-workers demonstrating that cytoskeletal and nuclear alignment promote spontaneous neural differentiation and that the effect could be combined with chemical cues to create a synergistic condition to further enhance the differentiation.

In conclusion, engineering substrates to induce desired cell phenotype and genotype is an important strategy of scaffold design for tissue-engineering applications. In the present study, we demonstrated that fluorinated graphene can be used to enhance cell adhesion and proliferation of MSCs, and it exhibits a neuro-inductive effect via spontaneous cell polarization. Fluorinated graphene films are found to be highly supportive of MSCs growth and the coverage of fluorine has significant effects on cell morphology, cytoskeletal and nuclear elongation of MSCs. Through a rapid and large-area ink-jet printing method, we also demonstrated enhanced neurogenesis via channel confined cell elongation in the absence of chemical factor stimulation. Our work shows that fluorinated graphene

Figure 4. (a) Schematic drawing of patterning MSCs by printing PDMS barriers on graphene films directly. (b) Optical microscope image of printed PDMS on fluorinated graphene film (scale bar = 50 μm). (c–e) The aligned growth of stem cell on graphene, PFG and FG with printed PDMS pattern, respectively (scale bar = 100 μm). (f, g) MSCs preferentially attached on the FG strips and their F-actin aligned (red) and expressed neural specific markers- Tuj1 and MAP2 (green) (scale bar = 50 μm). (h) Percentage of immunoreactive cells for Tuj1 and MAP2 on unpatterned and patterned FG strips. Note that the patterned FG strips induce higher expression of Tuj1 and MAP2 in the absence of retinoic acid. (n = 6, p < 0.05).
sheets, produced in large scaled and patterned, may be a viable platform for tissue-engineering applications.

**Experimental Section**

**Cell Culture:** Human bone marrow derived MSCs were obtained from commercial sources (Lonza) and cultured according to their recommended standard protocol. Details are provided in the Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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