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Using minimalist self-assembling peptides as hierarchical scaffolds to stabilize growth factors and promote stem cell integration in the injured brain

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Abstract

Neurotrophic growth factors are effective in slowing progressive degeneration and/or promoting neural repair through the support of residual host and/or transplanted neurons. However, limitations including short half-life and enzyme susceptibility of growth factors highlight the need for alternative strategies to prolong localised delivery at a site of injury. Here, we establish the utility of minimalist N-fluorenylmethyloxycarbonyl (Fmoc) self-assembling peptides (SAPs) as growth factor delivery vehicle, targeted at supporting neural transplants in an animal model of Parkinson's disease. The neural tissue specific SAP, Fmoc-DIKVAV, demonstrated sustained release of glial cell line derived neurotrophic factor (GDNF), up to 172 hours after gel loading. This represents a significant advance in drug delivery, since its lifetime in phosphate buffered saline (PBS) was less than 1 hour. In vivo transplantation of neural progenitor cells, together with our growth factor-loaded material, into the injured brain improved graft survival compared to cell transplants alone. We show for the first time the use of minimalist Fmoc-SAP in an in vivo disease model for sustaining the delivery of neurotrophic growth factors, facilitating their spatial and temporal delivery in vivo, whilst also providing an enhanced niche environment for transplanted cells.

1. Introduction

The restoration of damaged brain and spinal cord after acute injury or neurodegeneration is limited due to a lack of innate cellular repair mechanisms in the central nervous system (CNS). This limited capacity for neural regeneration and reinnervation has led to the research and development of cell replacement therapy

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(CRT).(Rodriguez, *et al.*, 2012) Whilst clinical trials have shown evidence of functional integration of new cells,(Lindvall and Odin, 1994) most research remains in the development phase due to inherent variability in grafting outcomes. Current research is largely focused on strategies to improve graft survival and reinnervation of the host brain in order to promote graft-host interactions and improve overall regenerative outcomes.(Lindvall and Odin, 1994)

One of the most promising strategies to enhance endogenous and transplanted cell survival is the delivery of growth factors. Neurotrophic factors including neurotrophic growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) are of particular interest for delivery to the damaged CNS.(Allen, *et al.*, 2013) These proteins have been shown to promote survival of various neural cell populations both *in vitro* and *in vivo,* and are therefore attractive proteins for providing protection after CNS damage.(Kauhausen, *et al.*, 2013, Wang, *et al.*, 2012, Wang, *et al.*, 2016) The success of neurotrophic factor delivery is constrained however, due to inherent properties of growth factors including their short half-life and susceptibility to enzymatic degradation.(Tayalia and Mooney, 2009) In addition, the blood-brain barrier (BBB) prevents large molecules from penetrating the CNS, making localised delivery of neurotrophic factors to a site of injury challenging.(Donaghue, *et al.*, 2014)

Current methods involve the use of catheters and pumps or multiple bolus injections of soluble growth factor directly to the site of injury.(Nagahara and Tuszynski, 2011, Ruozi, *et al.*, 2012) These technologies present significant issues due to their invasive

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nature as well as potential for off-target effects as a result of systemic delivery.(Tayalia and Mooney, 2009) Furthermore, these methods are expensive, as high concentrations of growth factor are required to achieve therapeutic doses. Therefore, there is a need for delivery mechanisms that can overcome these limitations and provide direct, controlled, localised delivery for improved tissue repair outcomes.

Recently, biomimetic tissue engineering scaffolds have been recognised as an important tool in this endeavour. By presenting grafted stem cells with a synthetic, trophic extracellular microenvironment, tissue regeneration can be enhanced and the cell behaviour such as migration and differentiation can be directed.(Discher, *et al.*, 2009) In addition to providing the physical requirements necessary to promote neural repair, biomaterials have also been engineered for the spatial and temporal delivery of proteins and small molecules to induce repair mechanisms through the activation of various cell surface receptors and the induction of intracellular signalling cascades.(Herrán, *et al.*, 2013, Nisbet, *et al.*, 2010, Oliveira, *et al.*, 2013, Pakulska, *et al.*, 2013, Roam, *et al.*, 2014, Tam, *et al.*, 2014, Wang, *et al.*, 2014)

For instance, we have shown that the covalent attachment of GDNF to electrospun poly-caprolactone (PCL) scaffolds promotes survival, migration, proliferation and neurite outgrowth of transplanted neural progenitor cells *in vivo.(Wang, et al., 2012, Wang, et al., 2016)* Stability of the attached proteins was also investigated and revealed that GDNF retained its attachment and biofunctionality after 120 days.(Wang, *et al.*, 2014) Additionally, temporal control can be integrated within biomaterial design for growth factor delivery. Wang *et al.* achieved sequential delivery of epidermal growth

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factor (EGF) and erythropoietin (EPO) using diffusion from a composite biomaterial system in a stroke animal model, resulting in improved tissue repair compared to sequential delivery using a catheter/minipump system.(Wang, *et al.*, 2013) These examples highlight the usefulness of biomaterials in the design of sophisticated growth factor delivery vehicles that can circumvent the limitations of traditional delivery mechanisms.

Here, for the first time we demonstrate the use of SAP scaffolds as an improved protein delivery tool with superior *in vivo* spatial and temporal delivery. We highlight the capacity of these materials to support the survival of grafted stem cells and improve tissue repair outcomes in a rodent model of Parkinson's disease (PD). Importantly, the designed SAP has multiple functionalities as it concomitantly presents the laminin-based peptide sequence IKVAV, known to promote neural differentiation and neurite elongation(Tysseling-Mattiace, *et al.*, 2008) at high density,(Rodriguez, *et al.*, 2013) whilst also providing nanofibrous structural support to transplanted cells, and a system for local growth factor delivery. GDNF was selected as the growth factor for delivery as it is known to promote survival and neurite extension of endogenous and transplanted dopamine neurons (the degenerating neuronal population in our PD model),(Kordower and Bjorklund, 2013) while ventral midbrain (VM) cell grafts were selected to provide replacement dopaminergic neurons.(Lindvall and Odin, 1994) The enhanced cellular response to this biomimetic system emphasises the utility of SAPs as a sophisticated platform for CRT. Importantly, we have also demonstrated the

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potential of these materials to stabilise growth factors, significantly extending their *in vivo* delivery.

2. Experimental

2.1 Solid phase peptide synthesis

Fmoc-DIKVAV was synthesised manually at 0.4 mmol scale by solid phase peptide synthesis (SPPS) using a rotating glass reactor vessel. All chemicals were purchased from Sigma-Aldrich except for Fmoc protected amino acids, Hydroxybenzotriazole (HOBt), O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and Wang based resins which were purchased from GL Biochem (China). 4 Å molecular sieves were used to dry any dimethylformamide (DMF) for a minimum of 2 hours before synthesis.

The synthesis was performed by stepwise deprotection and amino acid addition. Deprotection of the N-terminal Fmoc group of the resin-anchored amino acid was carried out using a solution of 20 % piperidine in DMF. Coupling of the subsequent amino acid was achieved by the addition of a solution of Fmoc-protected amino acid (2 mmol), HOBt (2 mmol), HBTU (1.92 mmol), and N,N-diisopropylethylamine (DIPEA) (4.8 mmol) in DMF (8 mL). The final Fmoc group was not removed and the final resin-bound peptide washed with ethanol before being placed under constant vacuum for two days. A Kaiser test was used for the detection of free amines to confirm successful deprotection and coupling.(Kaiser, *et al.*, 1970) After drying, the Fmoc-DIKVAV peptide was cleaved from the resin using trifluoroacetic acid (TFA)

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with 2.5% distilled water and 2.5% triethylsilane (TES). After two hours in TFA solution, glass wool was used to filter out the resin from the peptide/TFA solution. The peptide/TFA solution was then sparged with nitrogen gas to remove excess TFA until 5 mL of solution remained. This solution was precipitated in cold ether and washed five times. The precipitated peptide was dried under constant vacuum for two days, ground into a fine powder, and then dried again for seven days before use.

2.2 Fmoc-DIKVAV gel preparation

All Fmoc-DIKVAV hydrogels were prepared at 20 mg/mL. 100 µL of deionised water with 50 µL 0.5 M sodium hydroxide (NaOH) was used to dissolve 10 mg of peptide. 0.1 M hydrochloric acid (HCl) was then added dropwise, always while vortexing, until the pH of the solution reached 7.4 and self-assembly occurred. Typically, 100 – 150 µL of 0.1 M HCl was required to form the hydrogels. When the optimal pH was attained, Hank's buffered saline solution (HBSS) (Gibco) was added to bring the gel to the appropriate concentration (20 mg/mL). Purity of the Fmoc-DIKVAV peptide was determined as ~96% as confirmed via high-performance liquid chromatography (HPLC) and Mass Spectrometry (Figure S1). Fourier transform infrared spectroscopy, Circular Dichroism, Transmission Electron Microscopy and Rheology (Figure S3) were used to confirm assembly and mechanical properties as previously reported (Horgan, *et al.*, 2016, Li, *et al.*, 2015, Rodriguez, *et al.*, 2014).

2.3 GDNF unfolding transition and release profile

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For the GDNF unfolding transition profile, samples of 200 ng/mL GDNF in PBS were prepared, left in an incubator, collected at regular intervals and stored at -20 °C until analysis by ELISA. For the GDNF release profile, 100 µL of previously prepared Fmoc-DIKVAV hydrogels mixed directly with GDNF solution (R&D Systems) to a final concentration of 1000 ng/mL via vortexing for 3 minutes. The shear thinned hydrogels were then placed in a 96 well plate and allowed to re-assemble post protein inclusion. This resulted in full entrapment of the GDNF within the hydrogel as the entire volume was gelated. 200 µL of phosphate buffer saline (PBS) was placed on top of the gel and the plate left in an incubator (37 °C, 5 % CO_2). The PBS supernatant was collected at regular intervals, replaced with fresh PBS and stored in at -20 °C until analysed by enzyme-linked immunosorbent assay (ELISA).

2.4 Enzyme-linked immunosorbent assay

Detection of GDNF was performed by sandwich ELISA, using previously described methods.(Wang, *et al.*, 2014) In brief, a 96 well plate was coated in GDNF capture antibody (R&D systems) overnight at room temperature. The following day, the wells were washed with 0.05 % Tween20 (Sigma) in PBS (PBST) and blocked for one hour at 37 °C, 5 % CO_2 using 2.5 % gelatin (Sigma) in PBST. The wells were again washed and 100 µL of standard or sample placed in each well for two hours at room temperature. The wells were washed and 100 µL of primary antibody against GDNF (R&D Systems) placed in each well for two hours of incubation (37 °C, 5 % CO_2). The wells were again washed and 100 µL of secondary horseradish peroxidase (HRP)

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antibody against the GDNF primary was placed in each well and left to incubate for one hour (37 °C, 5 % CO_2). The wells were washed and 100 µL of AB colour solution (Substrate reagent pack, R&D Systems) was placed in each well for 20 minutes at room temperature. Finally, 30 µL of 1 M HCl was added to each well for 5 minutes before measuring absorbance using a spectrophotometric plate reader (TECAN Infinite® 200 PRO series) at 490 nm.

2.5 6-hydroxydopamine lesions

All animal procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research. Experiments were approved by the Florey Institute of Neuroscience and Mental Health animal ethics committee. Adult Swiss mice were housed on a 12 h light/ dark cycle with *ad libitum* access to food and water. Anaesthetised mice were placed in a stereotaxic frame where anaesthesia was maintained at 2 % isoflurane for the duration of surgery. Using previously established methods, a craniotomy was performed for unilateral microinjections of the DA neurotoxin, 6-hydroxydopamine (6-OHDA, 3 µg) into ventral midbrain (VM).(Parish, *et al.*, 2001)

2.6 Isolation of ventral midbrain neural progenitor cells

Adult female C57BL/6 mice were used as graft recipients while donor tissue for transplantation was obtained from time-mated mice expressing green fluorescent

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protein under the tyrosine hydroxylase promoter (TH-GFP), (Sawamoto, *et al.*, 2001). Note, TH is the rate limiting enzyme in dopamine synthesis and commonly used as a marker for DA neurons. In this instance TH-GFP expression was used to identify graft derived DA neurons and fibres (i.e. GFP+) from residual host DA neurons (GFP-). Animals were time mated overnight and visualisation of a vaginal plug on the following morning was taken as embryonic day (E) 0.5. Embryos at E12.5 were collected in chilled L15 media. TH-GFP+ embryos were selected, their brains removed and the VM dissected to isolate primary VM tissue. The tissue was incubated for 20 minutes in 0.1 % DNase and 0.05 % trypsin in magnesium and calcium HBSS. The tissue was then washed gently 3 times with HBSS, dissociated and the cells re-suspended in HBSS with 0.1% DNAse at 100 000 cells/µL.(Thompson and Parish, 2013)

2.7 Transplantation of E12.5 ventral midbrain neural progenitor cells

At 3 weeks post-lesioning, mice received implants of cells alone, cells + Fmoc-DIKVAV or cells + Fmoc-DIKVAV + GDNF (n = 7 per group). Cells were implanted ectopically into the striatum – the target tissue of VM DA neurons and common site employed for cell replacement in PD. Cells were mixed at a 1:1 ratio with HBSS or Fmoc-DIKVAV (± GDNF at 1 ng/µL) prior to implantation into the striatum. A total of 2 µL (100 000 cells) was injected, using a fine glass capillary, at the following co-ordinates: 1.0 mm anterior and 2.3 mm lateral relative to bregma, and at a depth of 3.2 mm below the dural surface.(Thompson and Parish, 2013) After 10 weeks, mice were

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killed by an overdose of sodium pentobarbitone (100 mg/kg) and transcardially perfused with warm saline followed by 4 % paraformaldehyde (PFA). Brains were removed, post-fixed for two hours in 4% PFA and cryo-preserved overnight in 30% sucrose solution.

2.8 Immunohistochemistry

Brains were coronally sectioned (40 μm at a 1:12 series) and immunohistochemistry against GFP (rabbit anti-GFP, 1:200, Abcam) was performed to amplify the GFP signal, using previously described methods.(Bye, *et al.*, 2012) The number of GFP+ cells, volume of innervation and innervation density measurements were carried out on a Leica microscope (Leica CTR6000) using LAS software and ImageJ and adopting previously established quantification methods.(Bye, *et al.*, 2012)

2.9 Statistical analysis

One-way ANOVAs with Tukey post-hoc tests were used to identify statistically significant changes between groups. The data was also transformed in order to obtain equal variance and the tests repeated for statistical analysis. Statistical significance was set at a level of $P < 0.05$. Data represents mean ± standard error of the mean (SEM).

3. Results and discussion

As laminin is a major component of the ECM in the CNS, the laminin-epitope sequence IKVAV, previously incorporated into a minimalist Fmoc-SAP system for the design of a

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shear thinning, nanofibrous hydrogel, was able to present the peptide at high density for CNS tissue specificity.(Modepalli, *et al.*, 2014, Rodriguez, *et al.*, 2014, Rodriguez, *et al.*, 2013) Before assessment of this minimalist SAP as a growth factor delivery vehicle in the CNS, we fully characterised the material properties of the hydrogel. Of note, and as previously reported,(Bruggeman, *et al.*, 2016) the incorporation of GDNF into the SAP hydrogel had no impact on the physical properties of the scaffold. See supplementary Figure S1 (confirming purity of the peptide) as well as supplementary Figure S3, where we confirm the π-β assembly and the mechanical properties of the hydrogels. (Modepalli, *et al.*, 2014, Rodriguez, *et al.*, 2014, Rodriguez, *et al.*, 2013)

3.1. Fmoc-DIKVAV impedes GDNF unfolding resulting in sustained growth factor release and functionality

We initially investigated the amount of soluble GDNF in PBS (**Figure 1**) to determine the level of protein unfolding and or degradation. After only 1 hour, the majority (~85 %) of GDNF in solution was undetectable. ELISA antibodies require the protein to be in its native state, and as such, are unable to detect the conformational epitope in any GDNF undergoing the process of protein unfolding. For this reason, from herein we will refer to difference in protein levels as 'protein unfolding', noting that this may also include some contribution from degradation. Irrespectively, we know from previous work that such conformational changes/degradation results in an inability to offer neurotrophic support such as survival and differentiation (Horne, et al., 2010, Wang, et al., 2014, Wang, et al., 2016). While immediate delivery, followed by the subsequent

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rapid unfolding and functional inactivation, may not be problematic in some contexts, delayed delivery is imperative in the context of cell transplantation, where newly implanted cells are vulnerable and require neurotrophic support. This is an immediate and key requirement; before additional growth factors, targeted at promoting differentiation and maturation, can even be considered. This highlights the issues associated with the short-term availability of growth factors in solution and the biological challenge for their delivery in CRT. The development of a delivery system that provides a prolonged reservoir of functional growth factor is required in order to achieve therapeutic benefit.

Following investigation of the GDNF unfolding transition profile, we examined the release of soluble GDNF from our characterised Fmoc-DIKVAV hydrogel in order to determine its ability to stabilise the growth factor within the hydrogel and therefore prolong GDNF availability by allowing ongoing release from the hydrogel. The hourly release profile showed ongoing release of GDNF, with a peak at 12 hours, at which time approximately 12 % of the total hydrogel-loaded GDNF had been released (**Figure 2** A & C). Persistent GDNF release continued over several days before decreasing to levels below the detection limits at 6 days (144 hours, Figure2 B) and showing no further cumulative increase (Figure 2D).

Importantly, the cumulative release profile emphasises the capacity for sustained release using the Fmoc-DIKVAV delivery vehicle. When delivered with Fmoc-DIKVAV, an increasing release of GDNF is observed, up until 2 days (48 hours),

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when release slows again, plateauing around 57 % of GDNF delivered from the hydrogel (Figure 2C & D). Relative to the control (i.e. the 1 hour lifetime before all of the GDNF had unfolded in solution) this demonstrates that consistent, gradual release out of the SAP was achieved, rather than an initial burst and rapid unfolding/degradation, which has limited therapeutic benefit.(Gomes, et al., 2012). We propose that sustained, persistent delivery was achieved by the co-assembly of the growth factor leading to its adsorption onto the peptide fibrils resulting in macromolecular association.(Williams, *et al.*, 2011) The adsorption of protein to the peptide fibrils resulted in a reservoir of growth factor that is protected from localised interstitial fluids such as cerebrospinal fluid in situ (or culture media in vitro). Consequently, adsorption prevents protein unfolding until the time at which they are released via diffusion. Previously, we have characterised this effect with other macromolecules such as fucoidan, observing their close association with the bundles of nanofibers, and our ability to stabilise soluble molecules within our minimalistic SAP hydrogels (Horgan, et al., 2016, Li, et al., 2017, Li, et al., 2016), however, this is the first reported demonstration of this process with molecules where the maintenance of structure is vital to their function.

3.2. Neurotrophic factor delivery using Fmoc-DIKVAV improves graft survival in a Parkinson's disease animal model

Previously, we have demonstrated the biocompatibility of Fmoc-DIKVAV when injected into the intact or injured rodent brain (Rodriguez, *et al.*, 2014). Transplantation of primary or pluripotent stem cell-derived cortical progenitors,

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together with the Fmoc-SAPs, resulted in viable grafts, that showed improved differentiation and innervation of host tissue, all whilst eliciting a minimal inflammatory response.(Rodriguez, *et al.*, 2014) Here, we advance research by investigating the capacity for Fmoc-DIKVAV to support transplanted cells in the parkinsonian brain and assess its capacity to act as a therapeutic growth factor delivery vehicle. Supplementary Figure 2 demonstrates the ability of the Fmoc-DIKVAV hydrogel to support transplanted cells with comparable inflammation (microglia and astrocyte activation) to cell only implants. The 6-OHDA PD lesion model was selected as it is a routinely used and well-characterised disease model in which to study cell replacement therapy and the benefits of neurotrophin delivery to both host and grafted neurons. GFP immunoreactivity confirmed viabile grafts, rich in DA (TH-GFP+) neurons, in all transplanted animals.

As well as playing a significant role in promoting neuron survival and differentiation, the IKVAV sequence has been shown to inhibit glial scar formation,(Tysseling-Mattiace, *et al.*, 2008) a key advantage for promoting neural regeneration and reinnervation *in vivo*. As such, this bioactive sequence is an ideal candidate for creating a biomimetic ECM environment for neural applications. This is highlighted by the increasing trend in cell survival observed for grafts in the presence of Fmoc-DIKVAV compared to cells delivered alone (Figure 3A). Previously, we have showed a modest increase in the number of GPF+ cells within grafts implanted into Parkinsonian mice in the presence, compared to absence, of soluble GDNF.(Wang, *et al.*, 2016) Here, we show that the incorporation of GDNF within our Fmoc-DIKVAV hydrogel, and its

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subsequent sustained delivery, resulted in an almost 2-fold increase in the number of GFP+ cells surviving after 10 weeks, compared to Cells+DIKVAV-SAP alone (Figure 3A).

The cell density of the graft core was also assessed to determine the extent of GFP+ cell migration (Figure 3B) and assess whether the presence of the SAP gel promoted or impeded the movement of cells within the graft site and surrounding host tissue. No significant difference was measured between cells alone versus the delivery of cells with Fmoc-DIKVAV ± GDNF, suggesting that the enhanced biomimetic environment did not alter the migration of grafted ventral midbrain (VM) cells (Figure 3B).

The capacity for innervation of the host tissue by VM grafted cells was also assessed. The volume of innervation, delineated as GFP+ fiber innervation within the host striatum, was reduced when cells were transplanted in the presence of Fmoc-DIKVAV alone (Figure 4A) with a corresponding increase in the cell density (Figure 3B). This could be due to the interaction of cell surface receptors with the IKVAV sequences, presented at high density within the nanofibrous scaffold, resulting in the localisation of grafted cells to the site of Fmoc-DIKVAV injection. When delivered with GDNF, however, the volume of innervation was comparable to cells transplanted alone (Figure 4A), suggesting that the prolonged release of GDNF from the gel was sufficient to overcome the restrictive growth observed with the hydrogel alone.

More detailed assessment of graft-derived GFP+ fibre density in the host tissue surrounding the graft was also examined using optical density measurements at two different fields of view (FOV) adjacent to the graft (Figure 4B). There was no apparent

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difference between the three groups, suggesting that the presence of Fmoc-DIKVAV and/or GDNF did not deter neurite extension and innervation of grafted VM cells into the host tissue (Figure 4 I & J). These results demonstrate the value of the minimalist Fmoc-SAP system as a platform technology that can be built upon to provide a higher level of tissue regeneration through the modulation of the local physical and trophic properties of the environment. This provides a significant tool for recapitulating the extracellular milieu in order to improve on current CRT strategies in the CNS.

4. Conclusion

The delivery of growth factors to enhance CRT outcomes has shown promise in promoting graft cell survival, reinnervation of the host tissue and overall endogenous tissue repair in the CNS. Here, we have demonstrated an ability to overcome these limitations by developing an Fmoc-SAP adjuvant scaffold as a protein delivery system to stabilise GDNF in its native state and provide sustained protein delivery. We have demonstrated the versatility of the minimalist peptide scaffolds developed highlighting the applicability of the system for a large variety of tissue engineering applications. This sophisticated biomaterial will provide an alternative, multifaceted treatment strategy for a variety of tissue engineering application as it delivers physical and biochemical support to transplanted/endogenous cells whilst also having the ability to temporally and spatially control and sustain the delivery of drugs. Here, as proof of concept, we have demonstrated the utility of Fmoc-DIKVAV hydrogels as a growth factor delivery vehicle *in vivo* in a Parkinson's disease/cell transplantation model,

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adding another level of functionality to SAPs, through the sustained release of GDNF. This establishes the sophisticated level of biomimicry that can be achieved using Fmoc-SAPs as a platform for the design of advanced materials in regenerative medicine.

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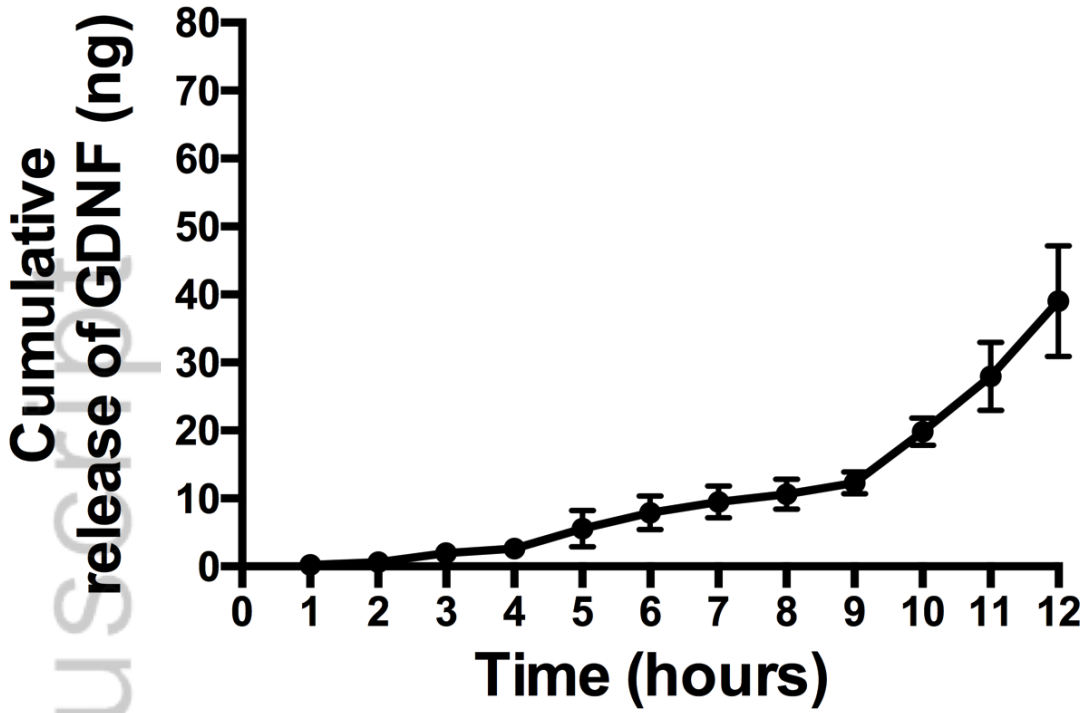

Figure 1

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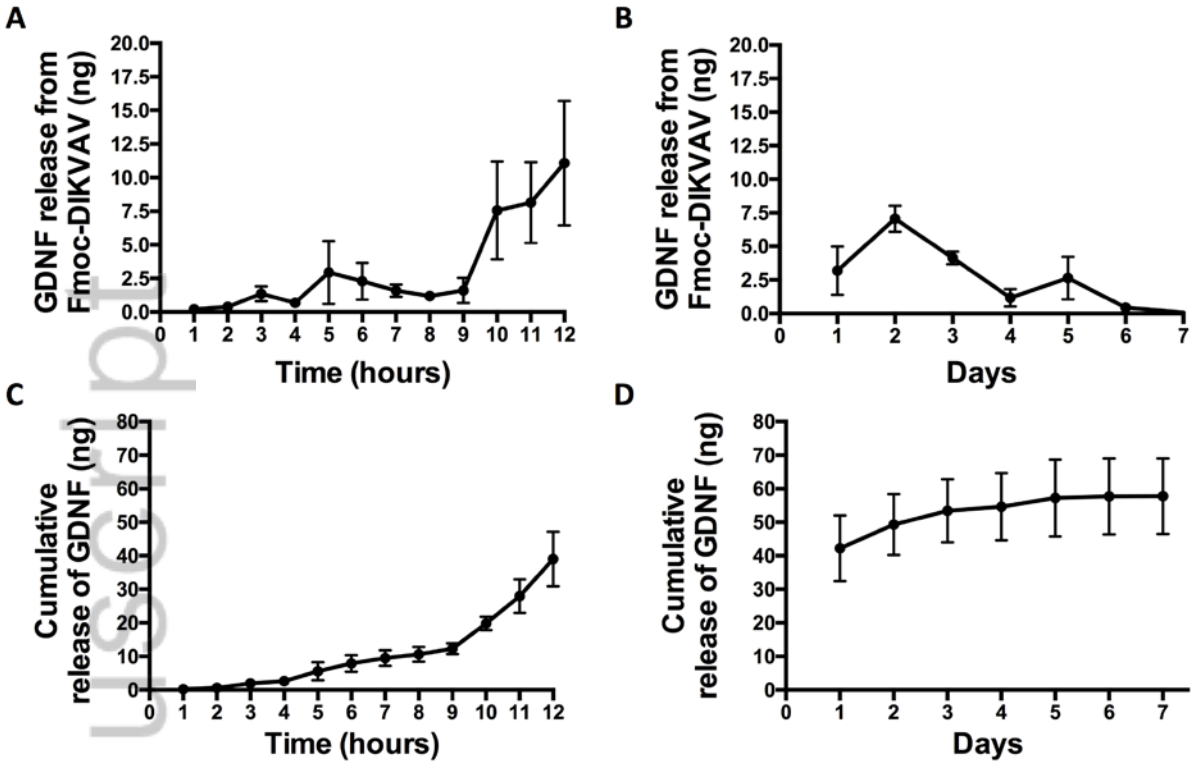

Figure 2

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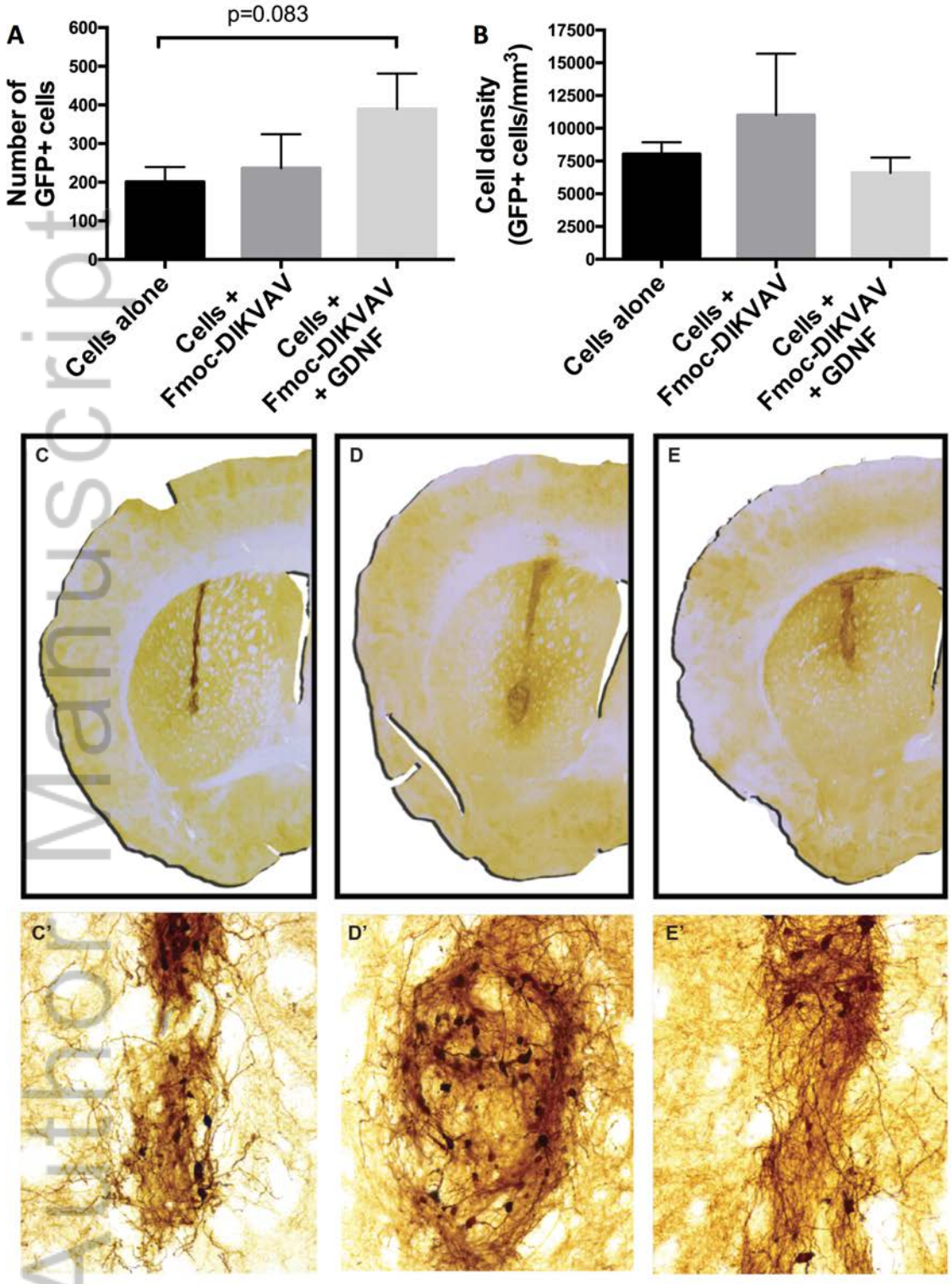

Figure 3

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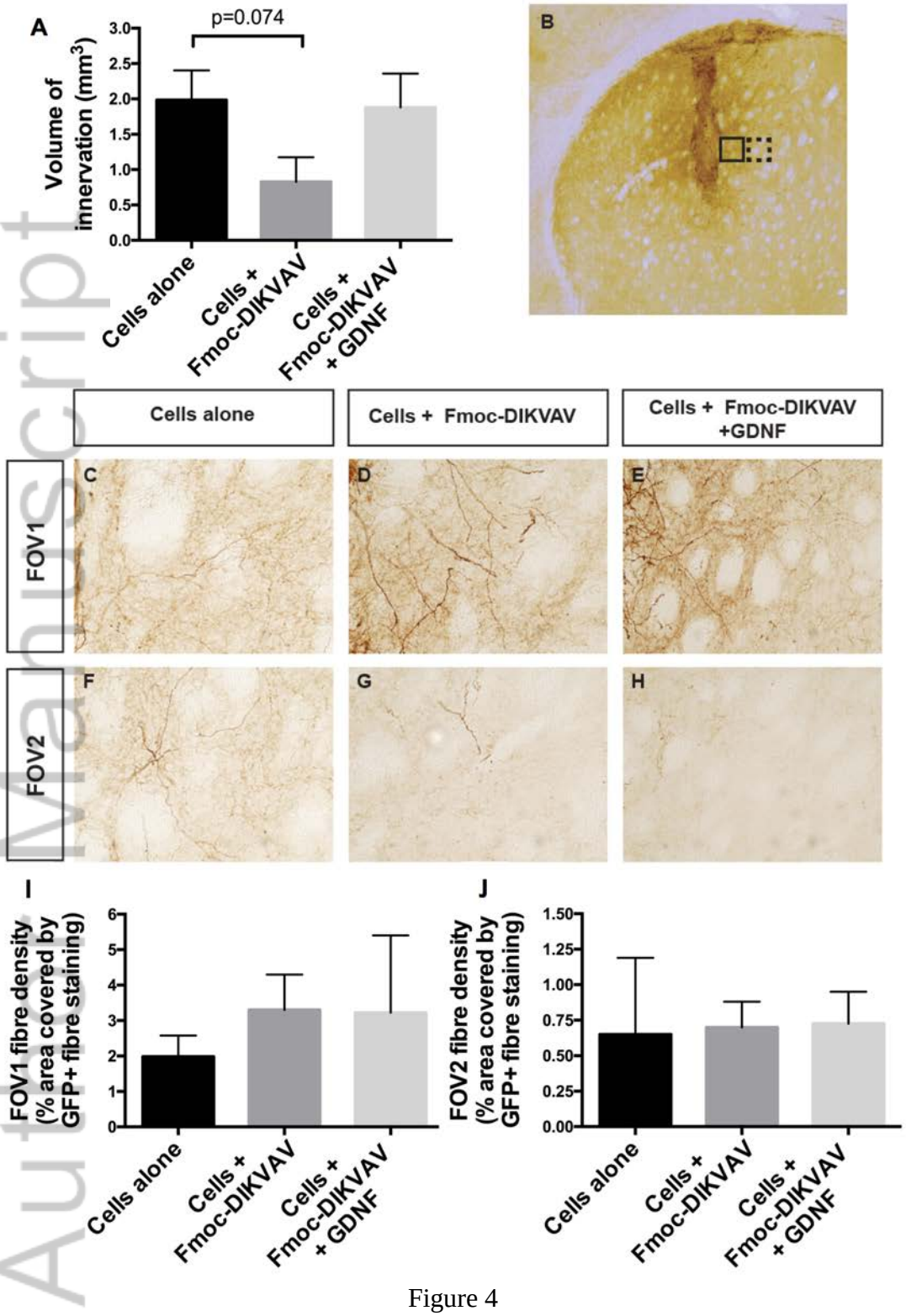

Figure 4

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