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The support of neural stem cells transplanted into stroke-induced brain cavities by PLGA particles

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ABSTRACT

Stroke causes extensive cellular loss that leads to a disintegration of the afflicted brain tissue. Although transplanted neural stem cells can recover some of the function lost after stroke, recovery is incomplete and restoration of lost tissue is minimal. The challenge therefore is to provide transplanted cells with matrix support in order to optimise their ability to engraft the damaged tissue. We here demonstrate that plasma polymerised allylamine (ppAAm)-treated poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) scaffold particles can act as a structural support for neural stem cells injected directly through a needle into the lesion cavity using magnetic resonance imaging-derived co-ordinates. Upon implantation, the neuro-scaffolds integrate efficiently within host tissue forming a primitive neural tissue. These neuro-scaffolds could therefore be a more advanced method to enhance brain repair. This study provides a substantial step in the technology development required for the translation of this approach.

Stroke is the most common cause of adult disability in industrialised countries [1]. Unfortunately, to date, no effective treatment is available to reverse the brain damage caused by stroke. Replacement of lost brain tissue by transplantation of neural stem cells (NSCs) is a potential therapeutic avenue that can offer some hope to patients [2]. Experimental studies in animal models have shown that NSCs can bring about some recovery of function [3] although recovery is never complete. In our own studies for example, NSC transplantation brought about a 30% reduction of lesion volume by 1 year following engraftment, but a large cavity of approximately 80 mm^3 remained [4]. In general, transplanted neural stem cells do not create *de novo* tissue, but integrate into the existing tissue matrix from which cells have been lost [3]. The lesion cavity, the void left by the lost ischaemic tissue, is void of all structural support and cells transplanted into this area typically migrate into surviving host tissue and show site-appropriate differentiation [3]. It can therefore be hypothesised that NSCs might improve tissue repair in the stroke-damaged area if sufficient structural support is provided within the lesion cavity. A more controlled delivery (i.e. defined number of cells per scaffold, amount of scaffold, reduction of carrier fluids or products), as well as a greater localised retainment of cells, may be achieved by supporting cells with a scaffold rather than introducing them as a simple suspension [5]. For instance, Park et al. [6] demonstrated that polyglycolic acid (PGA)-based scaffolds containing NSCs can enhance reciprocal interactions of donor and transplanted cells 7 days after a neonatal-induced hypoxia-ischaemia lesion. To date, this is the only demonstration of a transplantation of a scaffold complex into brain damage.

The challenges that emerged from this work are to design a biomaterial on which neural stem cells can attach and grow *in vitro* [7], but at the same time can be injected through a fine needle into the appropriate locale inside the brain to potentially completely fill the lesion cavity. Delivery of these neuro-scaffolds to the brain therefore needs to be image-guided to ensure that grafts are not injected into intact tissue, which may cause additional brain damage. Ideally, the engineered construct will also adapt to the diverse topology that is present within a lesion cavity. Creating individual particles loaded with neural stem cells on their surface here provides an advantage, as they can adopt the precise shape of the lesion cavity. At the same time, cells can maintain contact with other cells to create connections. This will also reduce the risk of a collagen-like response that could seal off the graft from host tissue. Interaction between host and grafted cells is further

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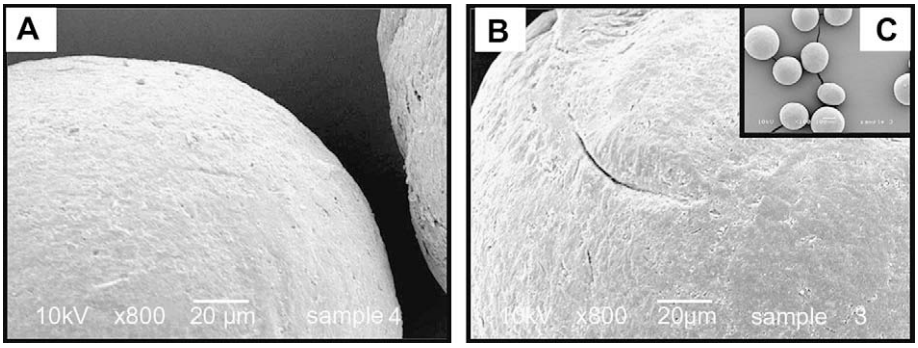

Fig. 1. Scanning electron micrographs of fabricated untreated PLGA microparticles at (A) ×800 magnification. (B) Microparticle following deposition with allylamine via plasma polymerization (0.5 KÅ layer, coating: incident power, 20 W; reflected power, <1 W) ×800 magnification. Microparticles at ×100 magnification (C).

facilitated by gaps in between particles for host fibres and blood vessels to invade the grafted area (i.e. void space). Gradually, the biodegradability of particles will further develop gaps and conduits (i.e. develop porosity) through which fibres and blood vessels can become established.

We here aim to 1) improve PLGA particle design to allow a large number of neural stem cells to be transplanted with structural support; 2) to use image-guidance for delivery into the lesion cavity; 3) fill the cavity using neuro-scaffolds; 4) use magnetic resonance imaging (MRI) to monitor the distribution of particles serially *in vivo*; 5) to determine if cells remained attached to the particles inside the cavity and 6) discover whether engrafted cells undertake differentiation into neurons and astrocytes that potentially could form *de novo* tissue. We describe a series of experiments that further each of these goals, and provide a neuro-scaffold with the potential to enhance NSC-mediated brain repair.

1. Material and methods

1.1. Microparticle formulation

Poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) microparticles were fabricated using a single oil-in-water (O/W) emulsion technique. The optimised method for the production of 100–200 μm-sized particles is described as follows. Briefly, 1.25 g PLGA polymer (85:15 PLGA, Lakeshore Biomaterials, USA) was dissolved in 5 ml of dichloromethane (DCM) at room temperature, overnight, in a glass scintillation vial. 5 ml of a pre-filtered 0.3% (w/v) polyvinyl alcohol (PVA) solution (Whatman No. 1 paper) was then slowly added to the vial before the solution was emulsified using a vortex mixer (VM20 vortex mixer, scale 4, 30 s). This primary O/W emulsion was then immediately poured into a beaker containing 800 ml of the pre-filtered 0.3% (w/v) PVA solution and stirred constantly (IKA magnetic stirrer, speed 3), for at least 24 h, in a fume hood to allow the solvent to evaporate. Following the complete evaporation of the solvent, the solidified microparticles were washed twice with distilled water and harvested via vacuum filtration using Whatman No. 1 filter paper. Microparticles were sieved and separated by a Retsch AS200 sieve shaker (amplitude: 1.40, 40 s interval time and 10 min total running time). For the 50–100 μm sized microparticles, a similar methodology was used, but with minor modifications: polymer concentration was adjusted to 1 g PLGA in 5 ml DCM, the primary emulsion created using scale 5, 45 s on the VM20 vortex mixer. The hardening bath was stirred constantly at speed 4. Long-term storage of microparticles was achieved using vacuum-sealed packaging and storage within a silica-filled desiccator. The size distribution of the pre-fractionated microparticles was determined by laser diffraction. A sample of approximately 50 mg of microparticles was dispersed in 13 ml of dH_2O. The suspension was then added to the chamber of a Coulter LS230 particle size analyser (Beckman, UK) under moderate stirring (default speed 5 setting) to the required concentration as indicated by the in-built display software. The particle size ranges were defined using PSS-Duke standards (Polymer Standard Service, Kromatek Ltd., UK). Particle size distribution was then determined as a function of the particle diffraction using the Coulter software (version 2.11a) and plotted as a function of volume percentage. Details regarding surface treatment via plasma polymerization can be found in the Supplementary Material. Scanning electron microscopy (SEM) was used to determine microparticle morphology, topography and size distribution. Microparticles were mounted on aluminium SEM stubs using double-sided carbon tape (Agar Scientific, UK) and then gold coated, for 3 min, under an argon atmosphere in a Balzers Union SCD030 sputter coater unit (Blazers Ltd., UK). Coated samples were then examined with a JOEL 6060LV variable pressure scanning electron microscope operating at an accelerating voltage of 10 kV. Image analysis was carried out using the in-built SEM Control User Interface software (version 6.57) and digital imaging system.

1.2. In vitro experiments

MHP36 cells [8] were grown at 33 °C in 5% CO_2 on fibronectin-coated (Sigma, UK) flasks. Harvested cells were pelleted at 900 g for 5 min and re-suspended in 1 ml of media. The required cell number (5×10^4, 1×10^5, 2.5×10^5, 5×10^5 or 1×10^6 cells/ml) was then added to 1 ml of media in individual wells ($n=3$ for each cell concentration and repeated 3 times) of a 24-well plate containing 20 mg of fibronectin-coated particles. Charged microparticles required fibronectin adsorption for adequate cell attachment. For this, fibronectin was diluted 1:100 of double-distilled H_2O and added to 20 mg of charged particles. Particles were re-suspended in this solution and the tubes placed on a shaker (Grant-Bio PMR-30, UK) at 30 rpm for 1 h at room temperature. To achieve a homogeneous cell attachment, shaking (5 min) and static (25 min) periods for the first 4 h of incubation at 33 °C were alternated and then left in motion at 30 rpm overnight at 33 °C in 5% CO_2. Cell counts of DAPI stained nuclei were obtained from 5 random images captured on a Zeiss Axioplan microscope (×10 objective). For average cell number/particle, DAPI-positive nuclei of attached cells were counted in one focal plane divided by the number of particles. For viability measurements, cells were Live/Dead® (2 mM Calcein-AM and 4 mM Ethd-D1, Invitrogen, UK) stained ($n=3$ wells, ×10 objective). The number of dead/live cells per field of view was expressed as a percentage of the total cell population. Cell proliferation counts were made on cells seeded onto microparticles in a parallel series of wells and stained only with a DAPI nuclear stain in vectashield. For stem cell marker expression, cells attached to microparticles were double-labelled for nestin (monoclonal mouse anti-nestin 1:100, Abcam) and SOX2 (polyclonal goat anti-SOX2 1:100, Abcam). A series of coverslips were seeded at 1×10^4 cells/300 μl and grown overnight before being stained for nestin and SOX2. Five random images/coverslip were captured using a ×5 objective.

1.3. Middle cerebral artery occlusion (MCAO) and transplantation

All procedures complied with the UK Animals (Scientific) Procedures Act (1986) and the Ethical Review Process of the Institute of Psychiatry. Twenty Sprague-Dawley rats (Harlan, UK) (weight 250–280 g at surgery) had their right MCA transiently occluded for 60 min by insertion of a propylene filament (Doccol Corporation, USA), as previously described [9]. Two weeks after MCAO, animals received transplants directly into the lesion at stereotactic co-ordinates determined from MR images (see below) with reference to the rat brain atlas [10]. Anaesthesia was induced by intraperitoneal (i.p.) injection of 0.25 ml/100 g of body weight of a mixture of 0.1 ml medetomidine hydrochloride (Dormitor, Pfizer, UK), 0.23 ml ketamine hydrochloride (Ketaset, Wyeth, UK), in 1 ml sterile H_2O. A 30 μl volume (=30 mm^3) of HBSS (Hanks balanced salt solution, Gibco, UK)/NAC (*N*-acetyl cysteine, Sigma, UK) containing microparticles with attached cells was injected directly into the lesion at a speed of 2 μl/min. MPH36 cells were prelabelled by incubation for 4 min with the fluorescent cell membrane marker PKH26 (Sigma, UK) before attachment to coated particles and subsequent transplantation. Cell viability before transplantation was 92%. No immunosuppression was given to animals [11].

1.4. Magnetic resonance imaging (MRI)

To determine the presence of a lesion after surgery and, if present, the extent and location of the lesion prior to transplantation, MR images were acquired 10 days after surgery using a 7.0 T horizontal bore magnet (Varian, USA) and a custom-built