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Tackling cell transplantation anoikis: an injectable, shape memory cryogel microcarrier platform material for stem cell and neuronal cell growth

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For some neurodegenerative disorders, particularly those such as Parkinson's disease which have a relatively selective loss of a specific cell type (dopaminergic neurons) from a relatively focal region, cell therapies offer a possible means of replacing or protecting the dying neurons.

Replacing the lost neurons by transplanting fetal ventral mesencephalic tissue into the striatum has shown clinical benefit,^[1] but some quite long-standing challenges still need to be resolved, particularly graft survival,^[2] as typically only 5-10% of the dopaminergic neurons survive the transplantation process.^[3]

Improving cell survival post transplantation to the brain is a research topic that has received much attention, with studies analyzing anti-caspase therapeutics, tropic support, gene delivery to the host site, and co-transplantation of supporting cells etc.^[4] These studies have resulted in limited success. In addition, a more recent approach of using materials such as soft hydrogels that gel *in situ* (*in vivo*) also showed only incremental improvements in cell survival.^[5, 6]

Whilst the host immune system clearly plays a significant role in determining the fate of the

graft,^[7, 8] there is mounting evidence that providing cells with a substrate on which to adhere prior to transplantation into the brain can lead to an increase in cell survival.^[9-11] Anoikis (Greek meaning: without a home^[12]), is the process by which cells die through lack of attachment, and has been proposed as a major factor contributing to cell death post-transplantation in the CNS.^[13] The process of using an *in situ* forming gel may affect the host response to the graft,^[6] but would not ameliorate the problem of anoikis as cells are added in a trypsinized state to the hydrogel precursor components. In contrast, microscale spherical poly-L-lysine coated glass beads have been used to culture cells on the surface prior to transplantation.^[11] Such a simple transplantation method produced a change in hippocampal neuron survival from reporting no surviving cells in the control group compared to a 76% cell survival when transplanted on glass beads. Such beads provided no barrier to the host response, no trophic support, and the animals received no immuno-suppression, thus showing it could be anoikis that was ameliorated by the provision an adherent surface throughout the transplantation procedure.

Such studies provide a solid rationale for the development of a more suitable material to which cells can adhere and differentiate, prior to implantation. For clinical application in the majority of CNS regions (excluding the spinal cord where macroscale scaffolds can be implanted^[14]) the scaffold material should be injectable^[15]. This means that the dimensions of the material must either be smaller than the inner diameter of the injection cannula/needle, or the material must be collapsible and mechanically tough, in order to withstand large deformation within the needle without losing integrity, and must re-expand to the original shape post injection. Recently, cryogelation technology was successfully applied for the preparation of macroporous hydrogel (cryogel) scaffolds.^[16-18] Cryogels are formed from the aqueous reaction mixture at sub-zero temperature using the process of ice crystal formation for structuring during the gelation process (**Scheme 1**) and subsequent lyophilization or melting of the ice crystal porogens renders a scaffold with a sponge-like structure. Due to

cryoconcentration, the non-frozen liquid regions taking part in gelation contain highly concentrated precursor molecules, resulting in cryogel struts which feature a higher stiffness compared to the corresponding bulk hydrogel materials.^[19] Furthermore, cryogels can withstand high compressive stress at high strains without destroying the gel matrix.^{[16][17][19]} The aim of this study was to use a recently developed biohybrid hydrogel material consisting of star-shaped poly(ethylene glycol) (starPEG) and heparin that already has proven biocompatibility in the brain^[20] to construct cryogel microcarriers for cell adherence/growth. We hypothesized that by using a combination of an emulsion technique with cryogelation, micron scale cryogel particles (microcarriers) could be produced which can bind growth factors, and allow cell growth in the interconnected macropores to protect them during injection through a small bore needle.

Cryogel microcarriers were synthesized via EDC/sulfo-NHS mediated crosslinking of amino terminated starPEG and Alexa 647 labeled heparin (**Scheme 1a**). After activating the carboxylic acid groups of the heparin, the crosslinking reaction took place in a water-in-oil emulsion. Therefore, small droplets of the hydrogel reaction mixture were formed in an organic phase by rapid stirring (end goal was to fit through a 27 gauge needle). The reaction vessel was then submerged in an ethanol bath at -20°C to facilitate ice crystal formation in the gelling mixture (**Scheme 1b**). Lyophilization removed both the surrounding organic solvent and the ice crystals from the gel matrix, to leave macroporous microcarriers ready for subsequent characterization and study.

SEM analysis of the dry cryogel microcarriers revealed that the microcarriers generally have a spherical form with a highly porous structure (**Figure 1a**). The pores are interconnected and could also be clearly seen present in the swollen wet state of the cryogel via CLSM (**Figure 1b**). During the transition from a dry state to a wet state, the cryogel microcarriers swell an average of 2.1 times in diameter, and 8.8 times in volume, which could be observed and calculated by light microscopy (see Supporting Information **Figure S1a**). The average

diameter of the cryogel microcarriers is 299 μm (**Figure S1b**) which is larger than the inner bore of a cannula typically used for transplanting cells into the brain (between 27-30 gauge, or 160 – 210 μm inner diameter).^[6, 21] However, the spongy materials exhibit irregular macropores with an average size of 72 μm (**Figure S1c**), separated by struts of only about 10 μm in width, and a high porosity, indicating plenty of spaces for collapse.

It is likely that anoikis is a large contributor to the vast cell death post transplantation to the brain; however, research groups have also investigated the idea of delivering neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) to the graft with the aim of improving survival. For example, poly(lactic-co-glycolic acid) (PLGA) spheres were used to deliver GDNF to the striatum during grafting of ventral mesencephalic (VM) tissue, but little beneficial effect was observed ^[22]. The same group then coated the PLGA spheres with poly-L-lysine and prior cultured the VM cells on the spheres before transplantation. A vast increase in cell survival was observed for cells transplanted on spheres either with or without encapsulated GDNF,^[10] showing that prior adherence is clearly important. We were attracted by the idea of providing a cell adherent microcarrier, which can also deliver growth factors to the transplant site, not just for graft survival but more for host neuroprotection. In this study we investigated GDNF and NGF for their neuroprotective properties.^[23] These growth factors were loaded to the microcarriers by electrostatic interaction between the proteins and the heparin^[20] within the cryogel microcarrier structure. Both GDNF and nerve growth factor (NGF) could be loaded onto the cryogel microcarriers at three different concentrations of loading solutions: 50, 100 and 500 ng/mL (see Supporting Information **Figure S2 and S3**). These three different concentrations gave distinctly different release rates of the growth factors into the surrounding medium, whereby the cryogels loaded with the most growth factor released the most growth factor over the seven day period (**Figure 1c, Figure S2b and Figure S3b**). Interestingly, it appears that NGF has a slightly greater affinity for the cryogel microcarriers as observed by the higher percentage loading and lower NGF release over the

seven day period than GDNF, which appeared saturated at the highest concentration, and showed a higher release rate.

Many spherical microparticles designed to deliver growth factors do so with an initial burst release, with the majority of the growth factor released over the first day or two.^[22-24] Indeed poor release kinetics is still a hurdle for local growth factor delivery in the CNS.^[23] In contrast, although the cryogel microcarriers are spherical in overall shape, the surface area of the cryogel struts (to which the growth factor is bound) is very different from that of a regular sphere. Furthermore, the highly negative charge of heparin allows for strong binding with growth factors such as GDNF^[25]. For example, PLGA spheres formed in emulsion,^[9, 26] or collagen spheres formed from a sacrificial template,^[27] consist of a shell of a surface area as opposed to the cryogel microcarriers which contain interconnected pores that reach right to the center of the particle.

Several cell types have been proposed for transplantation to the brain for restorative therapy of diseases such as Parkinson's disease.^[28] Within this study, we were focusing on bone marrow derived stem cells (MSCs) as they have been proposed not only for restorative therapy, but also for neuroprotective applications in the Parkinsonian brain as they can be readily transfected to over-express GDNF.^[29] However, despite promising rescue of dopaminergic neurons from neurotoxic insult in the vicinity of the graft,^[29] the poor survival of these cells *in vivo* limits their therapeutic potential.^[30] As a second cell type, the "neuron-like" cell PC12 cells were used in this study. To analyze the ability of MSCs and PC12 cells to attach and grow on the cryogel microcarriers, a spinner flask was used, whereby either cell type was added with the cryogel microcarriers into the spinner flask and stirred at 30 rpm with an on/off interval cycle of two minutes stirring, 30 minutes still, for the desired cultivation time (3 or 7 days). This culture protocol, often used for culturing cells on microcarrier materials,^[31] allowed for both cell types to attach to the cryogel microcarriers. **Figure 1d** shows a CLSM image of green fluorescent protein (GFP) fluorescent MSCs growing on a

cryogel microcarrier after three days in culture (also see **Figure S4a and b** and **Figure S8** for quantification of loading). When observing a thin section through the cross section of a cryogel microcarrier, the cells can typically be seen to be adherent to the outer edge of the sphere (**Figure S2a and b**), as this would be the first region the cell would contact during spinner flask cultivation. PC12 cells were cultivated for a longer time period (7 days) as it was desired to see if they could produce neurites on the cryogel microcarriers in the presence of NGF, which typically take an extended time to form.^[24] **Figure 1e and f** show CLSM images of PC12 cells grown in the presence of medium containing 500 ng/mL of NGF (non-differentiated PC12 cells can be seen in **Figure S4c and d**). For the NGF differentiated sample small neurites (**Figure 1e**) can typically be observed by β III tubulin staining, indicating that these cryogel microcarriers can support neuron growth prior to transplantation. In this way, it could be envisaged that neurons or iPS cell derived neurons could be allowed to differentiate, or partially differentiate as required, prior to transplantation without any trypsinization or axon damage prior to transplantation.

Interestingly we observed that when MSCs or PC12 cells were cultured on tissue culture plastic, or poly-L-lysine coated tissue culture plastic respectively, 24 hours after adherence both cell types partially migrated onto the empty (no cells or growth factor) cryogel microcarriers placed on top of them in the well. This could be quantified studying the cell metabolic activity after removal of the microcarriers, in comparison to untreated cells as the control group (**Figure 2a and b**). However, CLSM of the MSCs (**Figure 2c**) and light microscopy of the PC12 cells (**Figure 2d**) prior to the separation of the microcarriers from the original cell seeded well revealed that many cryogel microcarriers contained cells despite being originally added as empty cryogel microcarriers. Light microscopy revealed that with increasing concentration of cryogel microcarriers large clear patches could be seen on the well bottom, devoid of PC12 cells, whilst distinct cell aggregates appeared on the cryogel microcarriers (**Figure S5**). **Figure 2b** shows that after three days over 50% of the PC12 cells

had migrated onto the cryogel microcarriers at a concentration of 20 mg/mL (the highest concentration of microcarriers analyzed –almost a complete covering of the well bottom). The MSC migration was more varied (**Figure 2a**), however at all concentrations many cryogel microcarriers could be visualized to contain the GFP positive stem cells. For some applications, such as *ex vivo* gene delivery to the brain, integration of the grafted tissue is not important. For instance, in some clinical trials, integration has been purposefully avoided by the use of a protective encapsulating device to separate the transplanted tissue from the host cells and immune response, but still allow release of the cell secreted growth factor.^[32] However, for cell replacement therapy, such as with fetal dopaminergic neurons, graft survival and correct integration is of utmost importance for a functional benefit of the transplant, and remains a key research topic.^[33]

Poly-L-lysine coated glass beads provide a stiff surface for cell adhesion and migration^[11], but cannot be compressed upon injection so contain a large dead space and therefore limit the number of cells that can be transplanted.^[34] In contrast, the microcarrier system developed herein provides cells with locally stiff struts^[19] on which to adhere, yet allows a collapsible sponge-like structure for brain injection. Biomaterials designed for assisting cell transplantation or drug delivery to the majority of the brain regions must be injectable (except peripheral cortical regions^[35]), and, for minimal damage to surrounding tissue, fine gauge needles/cannula are often used.^[7, 36] Recently, neuronal networks have been formed on cryogel scaffolds, by seeding SHSY5Y cells or primary neurons on a preformed alginate and carboxymethyl-cellulose macroscale cryogel.^[18] Although cell survival was shown after injection of these scaffolds *in vitro*, a 16 gauge needle was required. This large gauge (needle outer diameter of 1.65mm) severely limits the analysis of such macroscale scaffolds in the midbrain of small rodents (for example the cross sectional mediolateral width of the striatum is typically around 3.3mm for 200-250 gram adult Sprague Dawley rats^[27]) as it would cause too much tissue disruption. For future cell transplantation applications in Parkinson's disease

models we wanted to analyze if the cryogel microcarriers could fit through a 27 gauge needle (inner diameter 0.22 mm, outer diameter 0.41mm) and yet maintain cell viability. **Figure 2e** shows the cell viability after the injection of microcarriers seeded either with rMSCs or PC12 cells, either using no needle (control group) or using a 27 gauge needle. The cryogel can collapse and reform outside the needle without affecting the overall shape of the microcarrier, and **Figure 2f** shows a sequence of images taken as cryogel microcarriers pass unaffected through the needle. No resistance was felt to injection, and after a several injections of unloaded microcarriers, no damage or small fragments could be observed. This is underpinned by the long plateau region in the uniaxial compression stress-strain curves of the corresponding bulk cryogel materials (**Figure S6** and References [17, 19]), demonstrating that the sponge-like materials are able to adsorb large amounts of energy without experiencing a large increase in stress. Consequently, these rather soft materials are very tough and do not even break at a compression strain of over 90%.

In conclusion, microscale macroporous hydrogels could be prepared using a water-in-oil emulsion and by allowing the subsequent gelation to occur in the aqueous phase at -20°C (cryogelation) and were hence termed cryogel microcarriers. These microcarriers could be functionalized with two different growth factors loaded with both MSCs and PC12 cells. Due to their spongy structure and their dense pore walls, that provide structural support to the highly porous materials, the cell loaded cryogel microcarriers could be injected through a 27 gauge needle without destruction and without loss of the cell viability. Moreover, it was shown that both cell types could migrate from the well plate onto the microcarriers suggesting that they will not hinder graft integration in the brain. Finally, the near zero order release profile of growth factors suggest that the cryogel microcarriers could be used for a combination therapy for neurodegenerative disorders. Future studies will assess whether the cryogel microcarriers can improve the survival of fetal ventral mesencephalic cells after

transplantation into the parkinsonian rat brain, where injection through a small bore cannula without backpressure or clogging will be vital.

Experimental Section

Cryogel Microcarrier Preparation:

The cryogels were prepared by crosslinking the amino end-functionalized starPEG with EDC/sulfo-NHS-activated carboxylic acid groups of heparin at -20°C. 200 µl of hydrogel mixture was prepared with a molar ratio of starPEG to heparin of 1:1 (11.11 mg and 15.56 mg respectively) and a ratio of EDC to sulfo-NHS of 2:1 (1.7 mg and 0.97 mg respectively). Therefore, heparin, starPEG, EDC and sulfo-NHS were dissolved separately in deionized, decarbonized water (MilliQ) using an ultrasonic bath filled with ice-cooled water for approximately 3–5 minutes, and afterwards all solutions were kept on ice. A two-fold molar excess of EDC to NH₂-groups of starPEG was used and added with the sulfo-NHS solution to the heparin. After vortexing (Minishaker MS2, IKA, Germany) and 15 minutes incubation on ice (to activate the heparin carboxylic groups) the starPEG solution was added and the mixture was further vortexed for 15 seconds.

A water-in-oil emulsion was used to create macroporous particles of the hydrogel. 200 µl of the hydrogel reaction mixture was added to 4 ml of toluene (the non-solvent medium containing Synperonic PEP105 as a stabilizing agent). The two-phase system was stirred (600 rpm) at room temperature for 13 minutes to form droplets. However, after 12 min, 1 ml of a higher concentrated Synperonic solution (20 mg/ml) was additionally added to prevent droplet agglomeration. The dispersion was chilled to -20°C with an ethanol bath cooled by an immersion cooling unit (TC 100E-F-NR, Huber, Germany). The frozen droplets were stirred for two hours at -20°C, before they were allowed to settle and remain overnight at -20°C. The microspheres were lyophilized overnight to remove the toluene and ice crystals. The microcarriers were washed three times with ethanol and two times with PBS.

Stem Cell Loading Studies:

Mesenchymal stem cells (MSCs) extracted from a GFP transgenic rat ^[30] were a kind gift from Dr. Linda Howard, National University of Ireland Galway, Ireland. They were cultured in a half/half mix of Minimum Essential Medium Eagle Alpha Modification (α MEM) and F12 media, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) and incubated at 37°C in 5% CO₂ at 90% humidity. For loading cells to the cryogel microcarriers, a spinner flask method was used, whereby the spin rate and the interval between spin and non-spin cycles can be controlled. The spinner flasks (glass, for a 25 ml volume, Chemglass Life Sciences, Germany) were first coated with Sigmacote® (Sigma, to reduce cell adhesion to the glass walls), and allowed to dry before autoclave sterilization. A mass of the cryogel microcarriers was obtained in the semi-swollen by weighing them out into a 40 μ m pores size well insert filter with excess liquid removed. A ratio of 50 mg semi-dry microcarriers per 1 million cells was kept constant throughout the experiments. The cryogel microcarriers were firstly re-hydrated with PBS containing 0.1% ProClin300 (Sigma) and left overnight for sterilization. After washing (three times) with sterile PBS, the cryogel microcarriers were then re-suspended in the same media used for culturing the MSCs and then added to the spinner flask. 1 million cells were then obtained by trypsinization from the culture flask, suspended in fresh media, and added to the spinner flask, to make a total volume of 25 ml. This was then placed in the incubator on top of a Variomag Biomodul 40B controlled magnetic stirrer (H + P Labortechnik GmbH), and set to a spin cycle of 30 rpm for 2 minutes with 30 minutes interval between spin cycles. After the desired incubation time, the cell loaded cryogel microcarriers were removed and again filtered to remove the excess media, before being processed for further study (e.g. fixation, microscopy etc).

PC12 Cell Loading:

PC12 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) GlutaMax supplemented with 5 % fetal bovine serum (FBS), 5% horse serum and 1%

penicillin/streptomycin (P/S) and incubated at 37°C in 5% CO₂ at 90% humidity. Cells were loaded to the microcarriers using the spinner flask method described above for the stem cells. However, this was performed either in the presence or absence of NGF in the media (500 ng/mL). Cells were visualized by CLSM after β III tubulin staining using a immunocytochemistry protocol adapted from Kraskiewicz et al.,^[24] but with a 3 μ m pore cell culture insert for easy separation of cryogel microcarriers from the solutions.

Effect of Injection on Microcarrier Shape and Cell Viability:

20,000 cells per mg of cryogel microcarrier were loaded as described earlier. After three days of cultivation at 37°C in 5% CO₂ at 90% humidity, 100 μ l of the microcarrier media suspension was injected into a 96 well plate via a sterile 1 ml syringe at a rate of approximately 1000 μ l per minute (as used by Aguado et al.,^[37]). The injection was carried out with or without a 27 gauge needle. After three hours of incubation following the injection process, the percentage of cell survival was analyzed using PrestoBlue[®] (a Resazurin-based assay), normalized to cells injected via the syringe without a needle (negative control).

Resazurin-based assays can be used to measure cell viability via the metabolic activity of the cells. PrestoBlue[®] (10 μ L) was simply added to the 100 μ L of cell loaded microcarriers that had either passed through the syringe and needle (test group) or just pushed out of the syringe (control group). After an incubation time of 30 minutes (37°C in 5% CO₂ at 90% humidity) the supernatant was removed and the fluorescence intensity was measured using a Tecan multiwell plate reader according to the manufacturer's protocol.

Statistical Analysis:

A student T-test was used for comparison of the cell survival during the injection procedure with and without a needle, ($P \leq 0.05$) using GraphPad Prism software.

Supporting Information

Supporting Information, including materials, cryogel microcarrier characterization, growth factor loading and release, and cell migration analysis is available from the Wiley Online Library or from the author.

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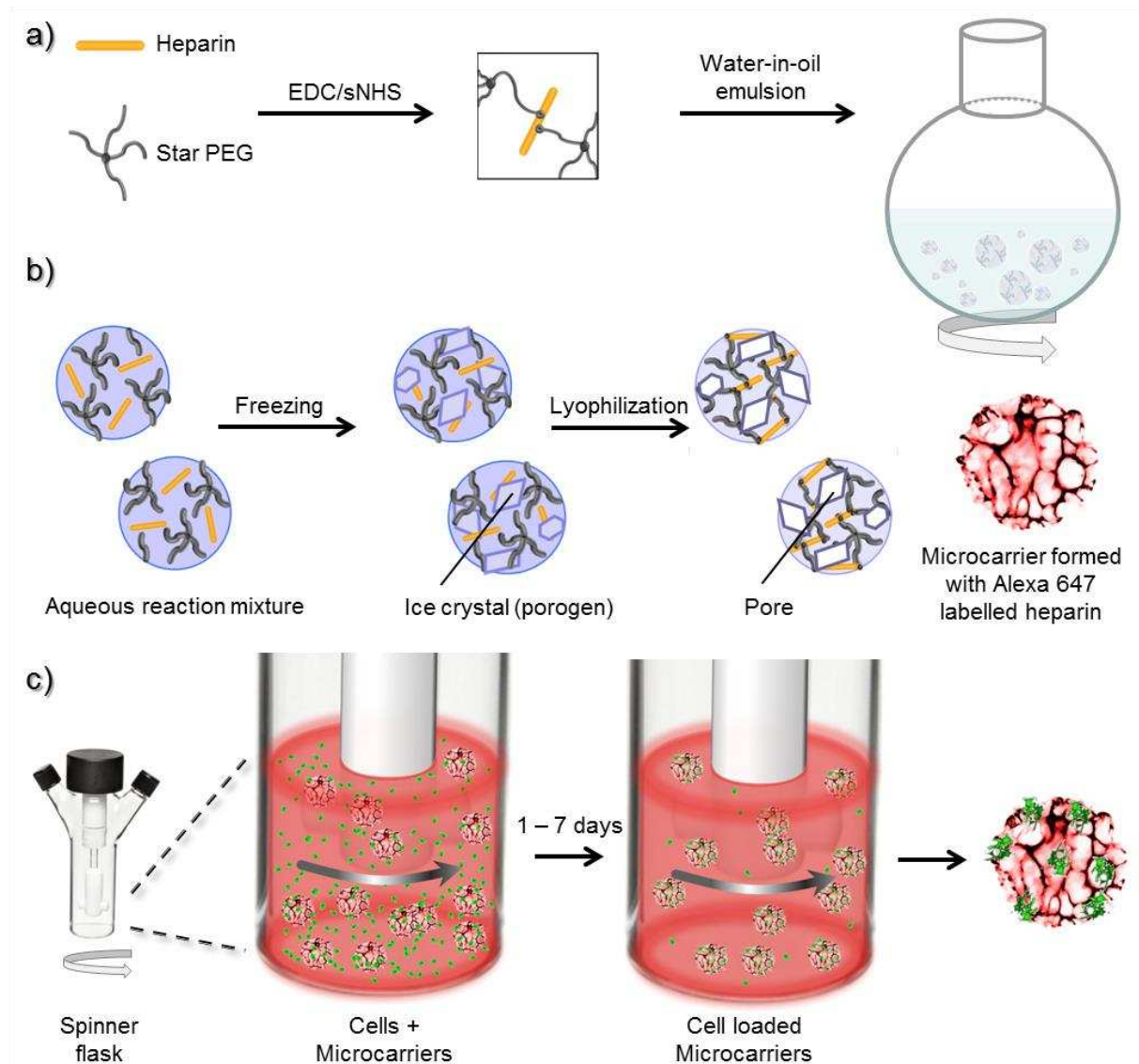
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Scheme 1. A schematic diagram depicting the synthesis and cell loading of the cryogel microcarriers. (a) StarPEG and heparin are crosslinked via EDC/sNHS chemistry in droplets of the aqueous reaction mixture in a non-solvent medium containing a stabilizing agent. (b) Freezing the gelling solution followed by lyophilization gives rise to the macroporous structure. (c) Cells can be cultivated on the cryogel microcarriers using a spinner flask culture method.

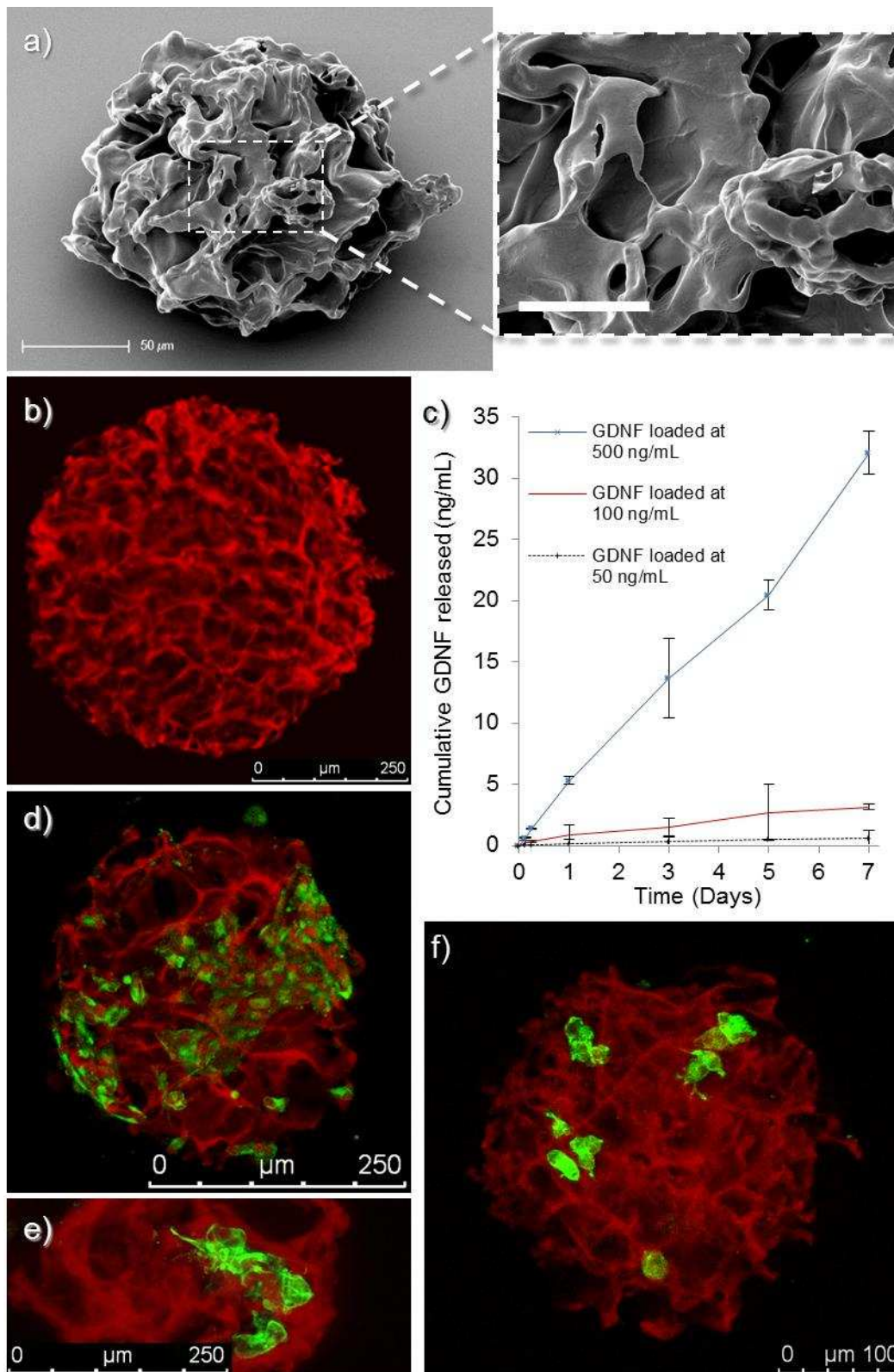


Figure 1. Cryogel characterization and cell growth. SEM image of the dry microcarrier (a) (and high magnification insert, scale bar: 20 μm) and CLSM fluorescence image (b) of Alexa 647 labeled PBS swollen cryogel microcarriers (red). (c) GDNF released into the media from the microcarriers is dependent on the initial loading concentration. Representative CLSM images of GFP positive mesenchymal stem cell (green) (d) or small neurites projecting from β III tubulin stained PC12 cells (green) (e) and (f)) cultured on the cryogel microcarriers for seven days.

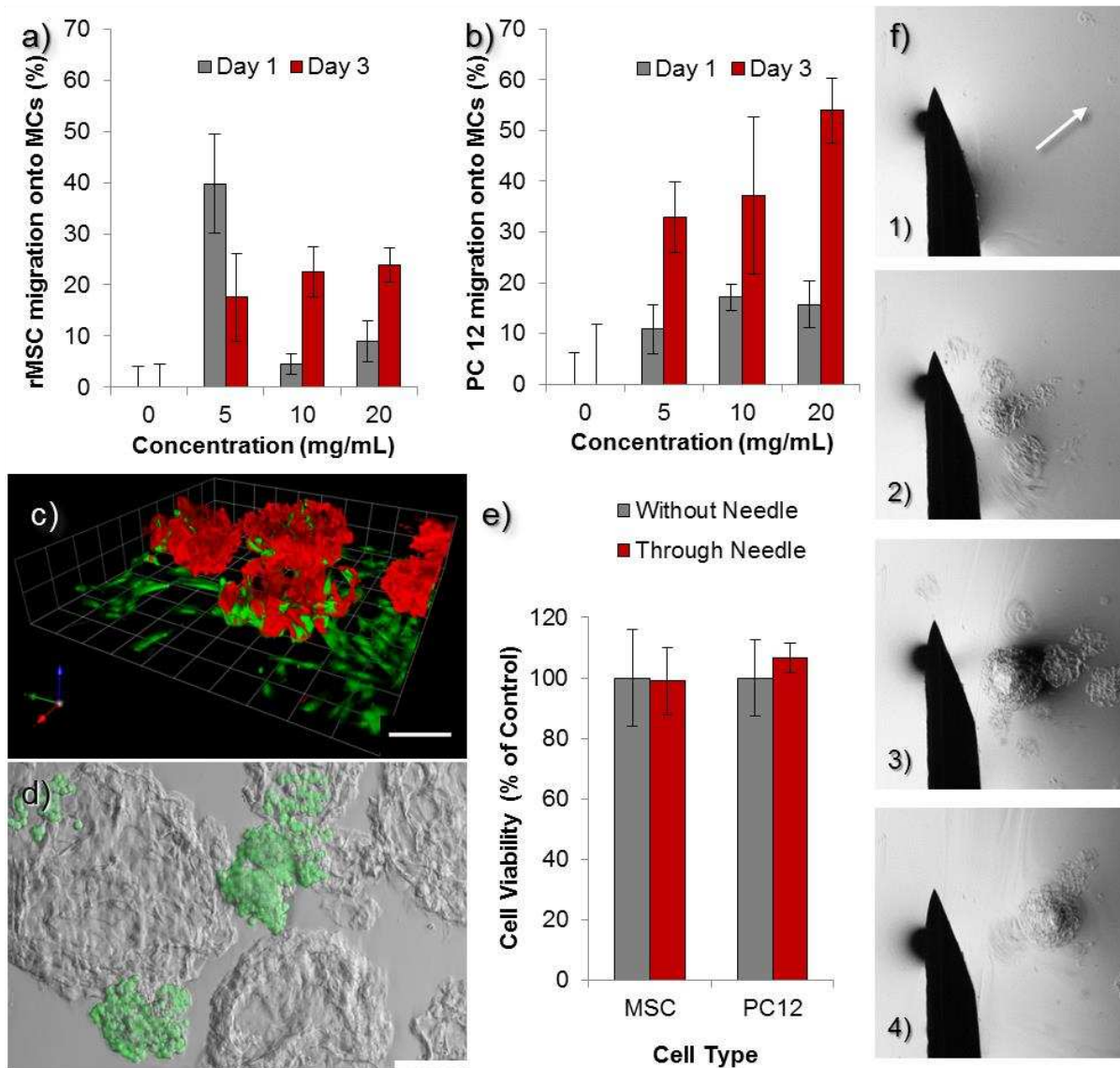


Figure 2. Cryogel microcarriers allow cell migration and injection through a 27 gauge needle. Quantification of rMSC migration (a) and PC12 migration (b) onto the cryogel microcarriers as a function of microcarrier concentration, as observed by CLSM for GFP positive MSCs (c) and light microscopy for PC12 cells (pseudo colored for clarity) (d). Injection of cryogels through a 27 gauge needle was shown to have no effect on cell survival (e) (n=4, no statistically significant difference between groups, student T-test ($P \leq 0.05$)) and could be visualized by light microscopy (see also Figure S7) (f)

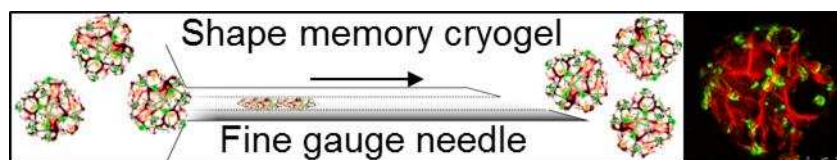
Table of Contents Graphic and Text:

Highly macroporous semi-synthetic cryogel microcarriers could be synthesized for culturing stem cells and neuronal type cells. Growth factors loaded to the heparin containing microcarriers showed near zero order release kinetics and cell loaded microcarriers could be injected through a fine gauge cannula without negative effect on the cells. These spherical microcarriers could therefore be applied for cell transplantation applications.

Cryogel

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Tackling cell transplantation anoikis: an injectable, shape memory cryogel microcarrier platform material for stem cell and neuronal cell growth



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