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Neurotropic growth factors and glycosaminoglycan based matrices to induce dopaminergic tissue formation

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ABSTRACT

Current cell replacement therapies in Parkinson's disease (PD) are limited by low survival of transplanted cell and lacking regeneration of neuronal circuitries. Therefore, bioartificial cell carriers and growth/differentiation factors are applied to improve the integration of transplants and maximize newly generated and/or residual dopaminergic function. In this work, biohybrid poly(ethylene glycol) (star-PEG)-heparin hydrogels releasing fibroblast growth factor 2 (FGF-2) and glial-derived neurotrophic factor (GDNF) were used to trigger dopaminergic tissue formation by primary murine midbrain cells *in vitro*. Matrix-delivered FGF-2 enhanced cell viability while release of GDNF had a pro-neuronal/dopaminergic effect. Combined delivery of both factors from the glycosaminoglycan-based matrices resulted in a tremendous improvement in survival and maturation capacity of dopaminergic neurons as obvious from tyrosine hydroxylase expression and neurite outgrowth. The reported data demonstrate that glycosaminoglycan-based hydrogels can facilitate the administration of neurotrophic factors and are therefore instrumental in potential future treatments of PD.

1. Introduction

The restricted ability of the brain to regenerate motivates research into restorative therapies and cell replacement strategies [1,2]. Within the group of neurodegenerative diseases, the treatment of Parkinson's disease (PD) with its progressive degeneration of midbrain dopamine (DA) neurons in the *substantia nigra* and the subsequent drastic decline of neurotransmission in the striatum became the focus of several promising restorative strategies. Intrastriatal transplantation of human fetal midbrain tissue in the human PD brain in double-blind, placebo-controlled clinical trials provided the proof-of-principle for DA neuronal replacement strategies, but also revealed negative graft-related side effects [3,4]. Alternative sources of DA neurons including embryonic or induced pluripotent stem cells are powerful but critically discussed due to the considerable risk for tumor formation. In this regard, fetal midbrain tissue accounts to be the 'gold standard' for intrastriatal cell transplantation into humans. A common limitation of grafting fetal midbrain cells is the poor survival of less than 10%, both in experimental animals and in humans [5–8]. The majority of grafted embryonic dopaminergic neurons die within the first 24 h post-transplantation [9]. Accordingly, improved survival of grafted DA neurons and axonal growth over long distances are major targets of ongoing research towards more effective cell replacement therapies for PD. Alternatively, and in combination with cell replacement strategies, boosting residual endogenous dopaminergic function of the diseased brain may offer additional options.

Neurotrophic factors – proteins that control survival, growth, differentiation and regeneration of the nervous system – have been implicated as potential therapeutic agents in the treatment of PD since they can exhibit cytoprotective and restorative functions on mesencephalic DA neurons. Glial derived neurotrophic factor (GDNF) was initially investigated to improve DA neuron survival and neurite outgrowth *in vitro* [10,11] and later shown to protect

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and restore the DA system of the lesioned rodent brain [11–13]. Apart from its neuroprotective function, GDNF is a restorative molecule and promotes formation of DA neurites [12,14,15]. Fibroblast growth factor 2 (FGF-2) improves dopamine neuron survival *in vitro* [16–19] and stimulates the neurite outgrowth *in vitro* [18] and *in vivo* [19]. Since both neurotrophic factors act through different receptors and signaling pathways [20,21], we expect additive effects by the combination of the two factors as reported earlier on dopaminergic cells [22,23].

Numerous strategies to deliver such neurotrophins *in situ* has already been used such as direct infusion using pumps [24], gene therapy for a virally mediated expression of proteins and transplantation of cells engineered to produce proteins [25,26]. Accordingly, several recent publications concern the development of customized polymer scaffold structures capable of supporting cell survival, alignment, maturation and functional integration of neural cells suitable for PD therapies [27–29]. Among those, materials for the delivery of neurotropic factors received particular attention. Poly(lactic-co-glycolic acid)-collagen microparticles were encapsulated with glial cell-derived neurotrophic factor (GDNF) fused with collagen binding peptide (CBP) and demonstrated to stimulate the transition of neural progenitor cells (NPCs) into mature neurons [30]. Combinations of vascular endothelial growth factor (VEGF) and GDNF released from poly(lactide-co-glycolide) microspheres were recently shown to be beneficial in the treatment of 6-OHDA-lesioned rats in the rotation behavior test and by pronounced tyrosine hydroxylase $(Th)^+$ dopaminergic neuron recovery [31]. However, as adhesion ligand presentation and physical features of the scaffold structures are additional cell-instructive parameters, biomaterials strategies enabling a far-reaching control over multiple characteristics are needed for the exogenous modulation of cells with neuroregenerative capacities.

For that purpose, we have recently reported a biohybrid hydrogel platform based on covalently cross-linked heparin and star-shaped poly(ethylene glycols) (star-PEG) in which network characteristics can be gradually varied while heparin contents remain constant [32,33]. The materials were shown to allow for the tailored covalent attachment of cell adhesion mediating peptides and the reversible, non-covalent binding of growth factors in ways resembling the presentation of factors in naturally occurring extracellular matrices. We applied the biohybrid gels to demonstrate the impact of mechanical and biomolecular cues on primary nerve cells and neural stem cells. The results demonstrated the cell type-specific interplay of synergistic signaling events and the potential of biohybrid materials to selectively stimulate cell fate decisions [32].

Now, we have further extended the options of this system by implementing customized release schemes for both the mitogenic factor FGF-2 and the pro-neuronal/dopaminergic factor GDNF, separately and in combination, aiming at improved survival of dopaminergic neurons as well as their maturation capacity and neuronal outgrowth.

2. Materials and methods

2.1. Preparation of starPEG-heparin hydrogels

StarPEG-heparin hydrogels were formed by crosslinking of the amino end-functionalized four-arm starPEG with EDC/s-NHS-activated carboxylic acid groups of heparin [32]. For this, a total polymer content of 11.6% and a 2:1:1 ratio of EDC:s-NHS:NH2-groups of starPEG [mol/mol] were used. In brief, heparin (MW 14,000, Calbiochem (Merck), Darmstadt, Germany) and starPEG (10,000 g/mol Polymer Source, Inc., Dorval, Canada) were separately dissolved in one third of the total volume of ice-cold deionized, decarbonized water (MilliQ) by ultrasonication and stored on ice. Afterward, EDC (Sigma–Aldrich, St. Louis, USA) and s-NHS (Sigma–Aldrich, St. Louis, USA) were each dissolved in the sixth part of the total volume of ice-cold MilliQ. After adding the EDC and s-NHS solution to heparin the solution were mixed well and kept on ice for 15 min for an activation of the carboxylic acid groups of heparin. Subsequently, the starPEG solution was mixed with the activated heparin for 15 min at 8 °C (at 900 rpm, Thermomixer Comfort, Eppendorf, Hamburg, Germany). The molar ratio of starPEG to heparin was 2. Surface immobilized networks with a final thickness of 50 μm were obtained by placing 3.11 μl of the gel mixture per cm^2 on freshly amino-functionalized glass cover slips for a covalent attachment of heparin through its activated carboxylic acid groups. In order to spread the gel solution equally on glass slides the gel solution was covered with a hydrophobic glass cover slip that has been treated with hexamethyldisilazane (Sigma–Aldrich) from vapor phase. For polymerization the gels were kept at 22 °C overnight (non-swollen gel mixture). Subsequently, the hydrophobic cover slips were removed and gels were washed in phosphate buffered saline (PBS, Sigma–Aldrich) to remove EDC/sulfo-NHS and unbound star-PEG/heparin. The PBS was changed five times every hour and once again after storage overnight. Subsequently, the swollen gels were immediately used for further experiments. For cell culture sterilization was performed by UV treatment for 30 min.

2.2. Biomodification of starPEG-heparin hydrogels

Biomodification with cyclo(Arg-Gly-Asp-D-Tyr-Lys) (RGD) peptide (Peptides International, Louisville, KY, USA) was performed as described in Ref. [32]. Briefly, surface-bound swollen hydrogels were washed in phosphate buffer (1/15 M, pH 5, 4 °C) 3 times. The buffer solution was exchanged with EDC/s-NHS solution (50 mM EDC, 25 mM s-NHS in 1/15 M phosphate buffer (pH 5), 4 °C) to activate the carboxylic acid groups of heparin. After 45 min, the gels were flushed 3 times in borate buffer (100 mM, pH 8, 4 °C) to remove unbound EDC/sulfo-NHS and immediately incubated in 0.2 mg/ml RGD-solution dissolved in borate buffer (see above) for 2 h at 22 °C. Finally, all samples were washed in PBS 3 times. For immobilization of FGF-2 or GDNF (both Sigma Aldrich) to the starPEG-heparin networks, PBS-swollen RGD-modified gels were immersed in the requested concentration (0.5 or 1.0 μg/ml) of growth factor solution at 22 °C for 6 h followed by washing in PBS for 1 min twice.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Immobilization and release studies of proteins by surface-bound starPEG-heparin hydrogels were performed as described in Refs. [34,35]. For the analysis surface-bound gels were placed in custom-made incubation chambers that allowed only minimal interaction of the protein solution with areas not originating from the hydrogel. 200 μl of FGF-2 or GDNF solution (both 1.0 μg/ml) were added separately or in combination per cm^2 scaffold area. Immobilization was performed overnight at 22 °C. Afterward, the gels were washed in PBS twice and the proteins were allowed to release from the gels at 22 °C into 250 ml/cm^2 of cell culture medium containing FCS (Gibco, Invitrogen, Carlsbad, USA) or without FCS, respectively at several time points and for a duration of 168 h. An equal volume of fresh medium was added back at each time point. Washing solutions and samples taken at intervals were stored at −80 °C until be analyzed by ELISA. Samples were assayed in duplicates using an ELISA Quantikine kit (R&D Systems, Minneapolis, USA) for FGF-2 or Emax® ImmunoAssay System for GDNF (Promega, Madison, USA) according to manufacturer's manual. Immunoassays for the