Biomaterials 136 (2017) 1–11

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Engineered hydrogels increase the post-transplantation survival of encapsulated hESC-derived midbrain dopaminergic neurons

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ARTICLE INFO

Article history:
Received 3 March 2017
Received in revised form
26 April 2017
Accepted 4 May 2017
Available online 5 May 2017

Keywords:
Dopaminergic neurons
Parkinson's disease
Transplantation
Cell replacement therapy
Hyaluronic acid

ABSTRACT

Cell replacement therapies have broad biomedical potential; however, low cell survival and poor functional integration post-transplantation are major hurdles that hamper clinical benefit. For example, following striatal transplantation of midbrain dopaminergic (mDA) neurons for the treatment of Parkinson's disease (PD), only 1–5% of the neurons typically survive in preclinical models and in clinical trials. In general, resource-intensive generation and implantation of larger numbers of cells are used to compensate for the low post-transplantation cell-survival. Poor graft survival is often attributed to adverse biochemical, mechanical, and/or immunological stress that cells experience during and after implantation. To address these challenges, we developed a functionalized hyaluronic acid (HA)-based hydrogel for *in vitro* maturation and central nervous system (CNS) transplantation of human pluripotent stem cell (hPSC)-derived neural progenitors. Specifically, we functionalized the HA hydrogel with RGD and heparin (hep) via click-chemistry and tailored its stiffness to encourage neuronal maturation, survival, and long-term maintenance of the desired mDA phenotype. Importantly, ~5 times more hydrogel-encapsulated mDA neurons survived after transplantation in the rat striatum, compared to unencapsulated neurons harvested from commonly used 2D surfaces. This engineered biomaterial may therefore increase the therapeutic potential and reduce the manufacturing burden for successful neuronal implantation.

1. Introduction

In recent years, pre-clinical advances have increased the potential of stem cell-based therapies for treating a range of human diseases [1], particularly for tissues that lack the capacity for robust regeneration from degenerative disease or injury, such as the central nervous system [1]. In particular, Parkinson's disease (PD) is a currently incurable, progressive neurodegenerative disorder characterized by motor and behavioral impairments that result in large part from a loss of striatal innervation by midbrain dopaminergic (mDA) neurons within the substantia nigra. PD affects approximately 1 in 800 people around the world and exerts a significant social and economic burden. Prevalent treatments for PD include dopamine agonists, such as Levodopa [2], and deep brain stimulation [3]. Unfortunately, Levodopa often wanes in efficacy due to desensitization, may lead to side effects such as dyskinesia, and is rarely successful in the long term [4,5]. Moreover, while deep brain stimulation can considerably alleviate motor symptoms, it does not alter disease progression and may be accompanied by intracranial hemorrhage and electrode-associated infections [6].

Replenishing mDA neuronal innervation, and thereby restore functions lost in PD, is a promising alternative. Fetal-derived

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http://dx.doi.org/10.1016/j.biomaterials.2017.05.008
0142-9612/

dopaminergic cells implanted in PD patients have yielded promising results [7–9]; however, this cell source has several disadvantages including poor availability, low purity, low reproducibility, and ethical complications [10,11]. Fortunately, impressive progress in the derivation of mDA neuronal progenitors from stem cell sources, in particular pluripotent stem cells (PSC), has led to successful preclinical results in animal models of PD [12–16]. These safer and more readily available cell sources offer considerable promise for clinical translation.

However, a major challenge for stem cell based replacement therapies in general, and mDA neuron generation and transplantation in particular, is low post-transplantation survival of implanted cells [17–19]. The number of surviving tyrosine hydroxylase (TH, an essential enzyme for dopamine production)$^{+}$ mDA neurons is only < 1–5% of the total cells implanted in preclinical models [12–14,20] or in clinical trials [21]. As anticipated, this low survival rate reduces the efficacy of cell replacement therapies and necessitates large numbers of functional mDA neurons to be generated. For example, to treat the estimated 1 million patients in the US alone, with a minimum of 100,000 dopaminergic neurons required to survive post-transplantation per patient [19], 1×10^{13} mDA neurons would need to be generated *in vitro* to account for ~1% post-transplantation survival. Scalable bioprocesses are being developed to increase the yield of mDA neurons generated *in vitro* [22,23], but on the other hand, new strategies are needed to improve their post-transplantation survival and thereby reduce manufacturing burden, while potentially increasing efficacy.

Several factors may underlie low post-transplantation survival of cells: i) mechanical and/or enzymatic stress during cell harvest, ii) mechanical stress during injection [24], iii) changes in the environment from 2D *in vitro* culture to 3D *in vivo* tissue [24,25], particularly when the latter is diseased, and iv) immune and inflammatory responses [26]. In prior studies, increased post-transplantation survival was observed when robust, immature stem and neural progenitor cells (NPCs) encapsulated within a 3D biomaterial matrix were injected, likely by alleviating issues ii and iii above [27–29]. However, for PD and other neurological disease targets, it may be desirable to implant more mature, lineage-committed neurons rather than NPCs, as the former can have a higher fraction of cells committed to the desired neuronal fate as well as reduce the risk of uncontrolled NPC proliferation [12,30]. Unfortunately, mature neurons including mDA neurons are typically more fragile than NPCs [31]. Moreover, when developing cell implantation as an approach to treat key disease targets such as PD, cell survival should be assessed at longer time points when disease symptoms are typically alleviated, such as 16–18 weeks [12,15], rather than the shorter term analysis typically conducted to date for NPC survival [27,32–34].

To investigate and address these survival challenges, we matured and transplanted mDA neurons encapsulated within an optimized 3D biomaterial platform. We first generated mDA progenitors within a 3D PNIPAAm-PEG gel, which can support large scale cell production, as previously described [22,23]. However, since the PNIPAAm-PEG polymer is non-biodegradable, we transitioned and further matured these mDA progenitors for several days in a rationally designed biodegradable hyaluronic acid (HA)-based hydrogel before transplantation. Maintaining cells in a 3D culture both avoided harsh conditions involved during harvesting from a 2D surface, which can damage the fragile neuronal processes and lead to reduced cell viability, as well as offered a protective environment during and after transplantation. HA – a readily available, biodegradable, naturally occurring polymer that is part of the extracellular matrix – is a favorable material choice for many biomedical applications [35–39] and has been successfully used to improve post-transplantation survival of encapsulated stem and progenitor cells [27,32,33]. Additionally, as it is fully-defined and brain-mimetic [40], HA may be a stronger candidate for a brain transplantation material compared to other options such as Matrigel and Alginate. However, HA has not yet been tested for the encapsulation, maturation, and engraftment of more delicate cells [31] such as hPSC-derived neurons. To address this need, we designed a tunable HA hydrogel based on bio-orthogonal click chemistry for rapid, non-toxic gelation under physiological conditions, that enabled 3D encapsulation of neural progenitors. Functionalizing the HA with an adhesive peptide (RGD) to promote cell adhesion [41] and adding heparin, a glycosaminoglycan with neurotrophic factor binding properties [42,43], increased dopaminergic differentiation and neurite extension in our 3D HA gels compared to non-functionalized gels, and also led to functional cells that fire action potentials. Finally, hydrogel encapsulation increased the post-transplantation survival of hESC-derived mDA neurons in the rat striatum ~5-fold compared to the current standard method of injecting unencapsulated neuron clusters. Optimized biomaterials therefore offer the potential to enhance graft survival while reducing cell manufacturing scale.

2. Results

2.1. Optimizing HA gels for mDA development

We first aimed to engineer a biomaterial platform to meet the following requirements (Fig. 1a): i) the capacity for fast, non-toxic gelation through bio-orthogonal crosslinking [44,45], ii) tunable stiffness tailored to support neuronal development [46], iii) the capacity for functionalization with bioactive ligands, iv) material stability for extended *in vitro* culture to allow neuronal maturation in 3D, and v) subsequent biodegradability and low immunogenicity [47] to facilitate injection *in vivo*. We therefore proceeded to optimize an HA hydrogel that could address these criteria. Although several HA gelation schemes have been developed previously [27,35,48], precisely tunable methods that result in rapid gelation under physiological conditions to allow encapsulation with low stress and high cell viability are not well-established. Here, we used Strain-Promoted Azide Alkyne Cyclo-addition (SPAAC) [44,45,49], which is a fast, catalyst-free, bio-orthogonal click reaction that proceeds to stoichiometric completion under physiologically relevant temperature and pH conditions. We first functionalized HA with dibenzocyclooctyne (DBCO) [50] (see Methods) and controlled the gel stiffness by tuning the HA-DBCO weight fraction and the degree of crosslinking via addition of different ratios of the PEG-diazide crosslinker. Through empirical optimization, we found a gel formulation with a storage modulus of ~350 Pa that supported culture of mDA neurons *in vitro* for up to 25 days. Importantly, complete gelation was achieved within ~5 min (Fig. 1b), which resulted in genuine 3D encapsulation of cell clusters and permitted rapid re-introduction of media to minimize cellular stress.

Next, we tuned the material to support mDA neuronal maturation, including functionalization via SPAAC with an azide-modified RGD-containing peptide and with DBCO-modified heparin. RGD incorporation has been shown to generally enhance cell adhesion and migration [41], and encourage axonal growth of non-mDA neurons [51,52]. Additionally, heparin binds several factors known to enhance survival of mDA neurons – such as glial-derived neurotrophic factor (GDNF) [53], brain-derived neurotrophic factor (BDNF) [54], pleiotrophin (PTN) [55], and fibroblast growth factor (FGF) [56] – and incorporation of heparin into transplanted biomaterials reportedly enhanced neurite sprouting in an injured spinal cord [57], primarily via controlled release of neurotrophic heparin-binding growth factors [58]. Thus, we hypothesized that inclusion of heparin in our HA gels could enhance maturation and