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Gelatin-based 3D conduits for transdifferentiation of mesenchymal stem cells into Schwann cell-like phenotypes

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ABSTRACT

In this study, gelatin-based 3D conduits with three different microstructures (nanofibrous, macroporous and ladder-like) were fabricated for the first time via combined molding and thermally induced phase separation (TIPS) technique for peripheral nerve regeneration. The effects of conduit microstructure and mechanical properties on the transdifferentiation of bone marrow-derived mesenchymal stem cells (MSCs) into Schwann cell (SC) like phenotypes were examined to help facilitate neuroregeneration and understand material-cell interfaces. Results indicated that 3D macroporous and ladder-like structures enhanced MSC attachment, proliferation and spreading, creating interconnected cellular networks with large numbers of viable cells compared to nanofibrous and 2D-tissue culture plate counterparts. 3D-ladder-like conduit structure with complex modulus of ~0.4 × 10^6 Pa and pore size of ~150 μm provided the most favorable microenvironment for MSC transdifferentiation leading to ~85% immunolabeling of all SC markers. On the other hand, the macroporous conduits with complex modulus of ~4 × 10^6 Pa and pore size of ~100 μm showed slightly lower (~65% for p75, ~75% for S100 and ~85% for S100β markers) immunolabeling. Transdifferentiated MSCs within 3D-ladder-like conduits secreted significant amounts (~2.5 pg/mL NGF and ~0.7 pg/mL GDNF per cell) of neurotrophic factors, while MSCs in macroporous conduits released slightly lower (~1.5 pg/mL NGF and 0.7 pg/mL GDNF per cell) levels. PC12 cells displayed enhanced neurite outgrowth in media conditioned by conduits with transdifferentiated MSCs. Overall, conduits with macroporous and ladder-like 3D structures are promising platforms in transdifferentiation of MSCs for neuroregeneration and should be further tested *in vivo*.

Statement of Significance

This manuscript focuses on the effect of microstructure and mechanical properties of gelatin-based 3D conduits on the transdifferentiation of mesenchymal stem cells to Schwann cell-like phenotypes. This work builds on our recently accepted manuscript in Acta Biomaterialia focused on multifunctional 2D films, and focuses on 3D microstructured conduits designed to overcome limitations of current strategies to facilitate peripheral nerve regeneration. The comparison between conduits fabricated with nanofibrous, macroporous and ladder-like microstructures showed that the ladder-like conduits showed the most favorable environment for MSC transdifferentiation to Schwann-cell like phenotypes, as seen by both immunolabeling as well as secretion of neurotrophic factors. This work demonstrates the importance of controlling the 3D microstructure to facilitate tissue engineering strategies involving stem cells that can serve as promising approaches for peripheral nerve regeneration.

1. Introduction

The application of cell-based nerve regeneration therapies, has been considered as a promising strategy for the treatment of large

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peripheral nerve injuries. Schwann cells (SCs), which form the myelin sheath around peripheral axons, produce extracellular matrix (ECM) molecules, integrins and trophic factors, such as NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), and GDNF (glial cell line-derived neurotrophic factor), to enhance guidance and support for regenerating axons. Therefore, SCs are considered an integral component of cell-based nerve regeneration therapies for peripheral nerve regeneration [1–9]. Although a number of studies have demonstrated enhanced axonal regeneration across nerve gaps using nerve conduits implanted with SCs [10–14], the limited availability, donor site morbidity and the slow *in vitro* growth of SCs restricts the clinical translation of this strategy [15]. As an alternative, in a few recent studies, *in vitro* differentiated mesenchymal stem cells (MSCs) possessing SC like properties have been explored in different types of conduits for nerve regeneration purposes [16–19].

Mesenchymal stem cells, isolated and derived from various connective tissue sources (e.g., bone marrow, adipose tissue, placenta, umbilical cord blood) [7], hold considerable potential for cell-based nerve regeneration therapies using autologous transplantation due to accessibility, plasticity, multipotent nature, differentiation ability into functional cell types, paracrine activity via secretion of bioactive molecules including neurotrophic factors such as, NGF, BDNF and GDNF [20,21] and lack of ethical concerns [15,22–27]. Among those sources, bone marrow-derived MSCs are considered as the most available cell type and designated as the gold standard, although MSCs derived from other sources (adipose tissue, placenta, umbilical cord blood etc.) have also shown promising potential for proliferation and differentiation into different cell types [28]. Various studies have suggested strategies for neuronal differentiation and *in vivo* transplantation of MSCs in order to treat peripheral nerve damage [29–32] and numerous benefits of using transdifferentiated MSCs have been reported including promotion of axonal regeneration, reduced lesion size, enhanced neuronal survival and improved functional outcomes [33–40]. The benefits of MSC differentiation for Wallerian degeneration and nerve regeneration also include superior *in vivo* viability, enhanced neurotrophic factor secretion and myelinating ability [41]. However, current difficulties in controlling the final fate of the implanted cell population and providing scalable differentiation protocols along with an ideal 3D matrix mimicking the ECM, limit the clinical use of MSCs [42,43]. The positive effects of undifferentiated MSCs, circumventing the limitations of differentiation procedures and clinical applicability, have also been reported. However, the transplantation of undifferentiated cells may cause *in vivo* differentiation of MSC into unwanted and non-neural cell lineages in response to local stimuli or other dominant cells in the area [41].

The differentiation of MSCs depends on multiple interacting factors in their microenvironment, including biological, chemical and physical cues which, in combination, result in a complicated differentiation behavior outcomes [44–51]. Recently, specific composition of the differentiation media, as well as mechanical properties of the ECM, such as structure, composition and elasticity, have been demonstrated to influence MSC differentiation [50,52–55]. For instance, matrix stiffness has been found to regulate the differentiation of MSCs into specific lineages indicating that softer substrates promote neurogenic, adipogenic and chondrogenic fates, while stiffer substrates enhance myogenesis and osteogenesis [45,51,56–61]. The dimension in which cells are cultured is also a crucial factor in determining the differentiation properties of the cells. The current understanding of most biological mechanisms, including differentiation, has been garnered from cells cultured on two-dimensional (2D) surfaces. Most studies examining MSC transdifferentiation have relied on 2D tissue culture plates, after which the cells are then seeded into various types of scaffolds for facilitating neural regeneration, leading to extra treatment steps, time, effort and cost [16,17,20,21]. The cells naturally exist in a complex ECM containing various components, mixed cell populations and cell-secreted factors, hence, employing a 3D culture model is more relevant to the physiological condition to explore cell-cell and cell-matrix interactions affecting transdifferentiation. Current knowledge with respect to the influences of mechanical and structural properties of 3D scaffolds on the transdifferentiation behavior of MSCs is significantly limited and should be expanded [62]. Most of the previous studies investigated the effect of matrix properties on the osteogenic differentiation of MSCs; on the other hand, only a few studies focused on the relationship between matrix properties and the differentiation of MSCs into SC like phenotypes [56,63–66].

Taking these motivations into account, in this study, gelatin based 3D porous conduits with ladder-like (LL), macroporous (MP) and nanofibrous (NF) structures were developed in order to directly transdifferentiate bone marrow-derived MSCs into SC-like phenotypes within the conduits for the treatment of large peripheral nerve gaps. This strategy was used to enhance the directed transdifferentiation of MSCs within a 3D conduit matrix and prevent unnecessary additional steps involving transdifferentiation in regular 2D cultures followed by implantation. Combined molding and thermally induced phase separation (TIPS) techniques were selected to manufacture different microstructured 3D matrices which are difficult to achieve by current electrospinning and self-assembly techniques [67,68]. The effects of conduit structure, morphology and mechanical properties on differentiation and paracrine activity of MSCs (secretion of neurotrophic factors such as NGF, BDNF and GDNF) were investigated, and the performance of 3D conduits were compared with their 2D cell culture plate counterparts. The paracrine activity of transdifferentiated MSCs in the conduits was assessed not only by detecting the released neurotrophic factors but also by measuring their biological activity through the evaluation of neurite extension on PC12 cells co-cultured with the MSCs across a porous membrane insert. The overall results indicated that the conduits prepared in this study with controlled structural properties can serve as promising candidates for 3D ECM platforms intended for nerve regeneration applications and can be further tested *in vivo*.

2. Material and methods

2.1. Materials

Type B gelatin from bovine skin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC), N-hydroxysuccinimide (NHS), (2-(N-morpholino) ethanesulfonicacid) hydrate (MES), Bovine serum albumin (BSA), all-*trans*-retinoic acid (ATRA) and β-mercaptoethanol (BME) were obtained from Sigma Aldrich. 1, 4-dioxane, acetone, ethanol were obtained from Merck. The cell culture media components, minimum essential medium (α-MEM) and L-glutamine were obtained from Gibco. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals and antibiotic-antimycotic was obtained from Invitrogen. The transdifferentiation components forskolin (FSK) and heregulin β1 (HRG) were purchased from EMD Millipore, while platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were obtained from Sigma and Promega, respectively. Triton X-100 and paraformaldehyde were supplied from Fisher Scientific. The primary antibodies, calcium binding protein Rabbit-α-S100 and Mouse-α-S100β were obtained from Abcam while low-affinity neurotrophin receptor Rabbit-α-p75 was obtained from Promega. The secondary antibodies, Donkey-α-Mouse-Cy3 and Donkey-α-Rabbit-Cy3 were purchased from ImmunoResearch while DAPI (4′,6-diamidino-2-phenylindole) was obtained from Invitrogen.