Differentiation of neural stem cells in three-dimensional growth factor-immobilized chitosan hydrogel scaffolds

Nic D. Leipziga,b, Ryan G. Wyliec, Howard Kimd, Molly S. Shoicheta,c,d,*

a Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada
b Department of Chemical and Biomolecular Engineering, University of Akron, Akron, OH, USA
c Department of Chemistry, University of Toronto, Toronto, ON, Canada
d Institute of Medical Sciences, Toronto, ON, Canada

1. Introduction

Recent advances in nanotechnology and tissue engineering afford exciting new regenerative medicine strategies. The principal goal of regenerative medicine is to promote tissue regeneration and healing after injury or disease [1]. This can be achieved through the delivery of cells and/or factors in a polymeric scaffold designed to provide a biomimetic microenvironment conducive to cell adhesion, proliferation, differentiation, and host tissue integration. Thus far, the major hurdle for tissue engineering is to create 3-D microenvironments that provide the multiple synergistic stimuli necessary to predictably guide cell behavior. This is especially true for scaffolds incorporating cells created for central nervous system (CNS) regeneration where physicochemical signals are vital for survival and proper cell function.

Currently available clinical treatments for diseased or damaged CNS tissue provide only minor pharmacological relief and sometimes minimize further degeneration [2,3]. One of the central foci of functional repair after spinal cord injury, for example, is aimed at restoring active connections between neurons, allowing the return of motor and sensory function. Current research is investigating how neural stem/progenitor cells (NSPCs) can be utilized for functional tissue engineering as a potential treatment for neurodegenerative diseases, stroke, traumatic brain injury and spinal cord injury [4,5]. Stem cell therapy offers an attractive area of research for potential medical therapies, especially for treating injuries in the CNS. To successfully utilize stem cells, we must be able to influence their migration, proliferation, and phenotype in a predictable manner. Currently, soluble biochemicals have proved most successful in altering NSPC function; however, this strategy is difficult to apply in vivo because of complexities involved in...
Spatially controlling dosing locale, maintaining constant concentration over time and mitigating cost. Most importantly, the ability to implicitly influence a specific phenotype through physical stimuli has not yet been discovered [6]. NSPCs are defined by their ability to self-renew through mitotic cell division and differentiate into astrocytes, oligodendrocytes and neurons [7–9]. These stem cells exist in specialized microenvironments or niches in the mammalian brain and are able to generate a limited number of neurons and glia [10,11]. The physicochemical cues NSPCs require for proliferation, migration and differentiation are beginning to be understood [12]; however, significant work remains in terms of their incorporation into 3-D scaffolds for regenerative strategies.

The role of soluble factors in guiding stem cell function, such as self-renewal and fate decision, has been widely studied [7,13] and has recently shown that IFN-γ internalization signal transduction to be studied [31]. We have a nascent understanding of the ligands and function [23] to implicitly in spatially controlling dosing locale, maintaining constant concentration over time and mitigating cost. Most...
using an absorbance measurement at 412 nm and comparing it to a calibration curve of serially diluted cysteine standards (Sigma).

Next, lyophilized sulfhydryl-MAC was dissolved in PBS (pH 7.4) at 3 wt% m/v with 20 mM tris-(2-carboxyethyl)-phosphate hydrochloride (TCEP; Sigma). 10 μM maleimide-streptavidin (Sigma) was added and allowed to react by shaking overnight RT. 10 mM maleimide-GRGDS or maleimide-K(FAM)GRGDS (AnaSpec, San Jose, CA, USA) was next added and allowed to react by shaking overnight RT. The resulting mixtures were then placed in dialysis membranes (12,000–14,000 MWCO; SpectraPor) and dialyzed against PBS for 3–0 d with 3 changes per d. The amount of immobilized K (FAM)GRGDS was determined by fluorescent measurement (485 nm excitation, 520 nm emission) with comparison to a standard curve of maleimide-K(FAM)GRGDS.

**2.5. Biotin–rIFN-γ immobilization**

For quantification of protein immobilization, biotinylated rIFN-γ was fluorescently labeled with 5(6)-carboxyfluorescein, succinimidyl ester (5(6)-FAM, SE; Biotium, Hayward, CA, USA). Labeling was performed by adding thirty times molar excess of 5(6) FAM-SE and allowing the reaction to mix for 4 h. The reaction mixture was placed in a dialysis cartridge (10,000 MWCO; Thermo-Fisher) and dialyzed against PBS for 2 d with 3 changes per d. Protein concentration was determined via absorbance measurement after dialysis was completed.

Immobilization to modified MAC was carried out by adding the desired concentration of biotin–rIFN-γ and mixing for 4 h to allow binding to MAC-streptavidin. For quantification experiments, fluorescent biotin–rIFN-γ was mixed with 1 wt% streptavidin conjugated MAC in PBS to achieve final concentrations of 50, 100, and 200 ng/mL of biotin–rIFN-γ. A control for non-specific adsorption was included by reacting MAC-SH (no streptavidin) with 200 ng/mL fluorescent biotin–rIFN-γ.

Hydrogel scaffolds were formed by adding the photoinitiator 1-hydroxycyclohexyl phenyl ketone (IRG-184; Sigma) dissolved into 100% ethanol at 300 mg IRG-184 per mL ethanol. The photoinitiator solution was sterile-filtered through a 0.2 μm filter, added to the MAC solutions at a concentration of 1.5 μl IRG-184 per g MAC and mixed/deaerated (SpeedMixer DAC 150 FVZ; Hauschild Engineering, Hamm, Germany). 100 μl of the resulting mixture was transferred into a 96-well plate and crosslinked for 2 min by UV light (365 nm) exposure. The resulting gels were washed...
3 times per d for 3 d with PBS to remove unbound protein. Mechanical testing of the hydrogels (Mach-1 micromechanical testing system, BioSyntech, Laval, QC, Canada) yielded a Young's modulus ($E_Y$) of 0.77 ± 0.18 kPa which is similar to our previous studies [6,23] and native brain tissue [36,37].

2.6. NSPC 3-D culture in immobilized rIFN-γ methacylamide-chitosan hydrogels

Cell culture experimentation was performed similarly to above. MAC-1 (streptavidin, GRGDS) in PBS was first sterilized by UV light for 2 h then solutions were made incorporating final concentrations of 100 ng/mL biotin-rIFN-γ (immobilized group), 100 ng/mL biotin-rIFN-γ (soluble group) and blank controls (control group). $2 \times 10^5$ cells/mL passage five dissociated NSPCs in growth medium and 1.5 μL Biotin-184 solution per g total mass of solution were added to each group to achieve a final 1 wt% hydrogel scaffold (g modiﬁxation and bioactivity of rat biotin tagged IFN-γ cultures expressing AviTag-rIFN-γ). Finally, cell nuclei were counterstained with 10 μM Hoechst 33342 (Invitrogen) for 7 min, washed with PBS buffer and mounted/coverslipped using ProLong Gold anti-fade reagent (Invitrogen).

2.8. Statistics

All statistical analyses were performed using JMP IN 7.1 (SAS Institute, Cary, NC, USA). Differences among groups were assessed by ANOVA with Tukey’s post-hoc analysis to identify statistical differences among three or more treatments. All data are presented as mean ± SD.

3. Results

3.1. Recombinant protein expression, characterization and bioactivity

In this study, BL21(DE3) E. coli were transformed with the Avi-Tag−rIFN-γ pET21a (+) plasmid and 4 h of IPTG induced production were sufficient to generate enough fusion protein for all experiments. Protein electrophoresis by SDS-PAGE (Fig. 2A) demonstrated that the expressed AviTag-rIFN-γ protein primarily resided in the insoluble fraction (inclusion bodies) of the BL21 (DE3) E. coli main culture. To obtain AviTag−rIFN-γ, the harvested E. coli pellet was completely denatured then purified utilizing the N-terminal 6X histidine tag. Optimally, 2 mL of Ni−NTA resin should yield a total of 80−100 mg of denatured protein according to the manufacturer’s specifications. Since only a few mg were required for experimentations, renaturation (or refolding) by dialysis was used to generate the native structure. Renaturation was initially performed by

**Fig. 2.** Recombinant production, purification and bioactivity of rat biotin tagged IFN-γ. (A) SDS-PAGE protein electrophoresis of insoluble (Avi−rIFN-γ insol.) and soluble (Avi−rIFN-γ sol.) fractions of E. coli cultures expressing AviTag−rIFN-γ. (B) FPLC with Superdex 200 results in one main peak that was selected for downstream use. (C) Bioactivity was assayed after biotinylation/purification with NSPCs seeded onto laminin-coated glass coverslips given 100 ng/mL of both soluble biotin−rIFN-γ and commercial rIFN-γ. After 8 d cells were fixed and stained for the neuronal marker βIII tubulin and the number of positive cells were quantified. No significant difference in neuronal differentiation was observed ($p = 0.94$). Data are presented as mean ± SD, $n = 3$. (D) Representative fluorescence images of βIII tubulin and Hoechst 33342 staining are shown.
dialysis in a Tris buffer with 0.2 M guanidine buffered at pH 7.5, which is sufficiently below the estimated isoelectric point (pI) of the fusion protein, \( pI = 8.64 \). This resulted in a small 2.00 mg yield of renatured AviTag eIFN-\( \gamma \) from a 1.8 L main culture. The addition of 0.5 M L-arginine to the dialysis buffer increased the renatured yield to 7.02 mg. As shown in Fig. 2B, FPLC purified samples resulted in a single large peak, demonstrating purity of the sample. MALDI-TOF of renatured and FPLC purified AviTag eIFN-\( \gamma \) had a molar mass of 21.41 kDa, which is similar to the theoretical molar mass of 21.35 kDa (Supplementary Table 1 DNA sequence 5′-3′ translation).

The FlowReporter Biotin Quantitation assay determined that AviTag-eIFN-\( \gamma \) was successfully biotinylated by the BirA enzyme with 102.7 ± 3.7% of eIFN-\( \gamma \) containing biotin. While the average is slightly above 100%, small errors may be the result of dialysis, transfer and pipetting steps. Before covalent attachment to MAC, the bioactivity of biotin-eIFN-\( \gamma \) was tested using 2-D in vitro culture on laminin-coated glass coverslips and compared to a commercial eIFN-\( \gamma \). We have previously shown that NSPCs robustly differentiate when exposed to 100 ng/mL of soluble eIFN-\( \gamma \) [22,23] on laminin-coated surfaces. IHC for βIII tubulin with positive cell counting demonstrated similar neuronal differentiation percentages for our E. coli-expressed eIFN-\( \gamma \) fusion protein and commercially available eIFN-\( \gamma \) (80.4 ± 2.4% vs. 80.2 ± 2.3% respectively; Fig. 2C, D; \( p = 0.94 \)).

3.2. MAC modification and covalent protein attachment

Maleimide-sulfhydryl chemistry was selected to covalently attach streptavidin to MAC in an aqueous solution through stable thioether bond formation. To enable maleimide-sulfhydryl cross-linking, first sulfhydryl-MAC was synthesized via the reaction with 2-iminothiolane. After purification, Ellman’s assay determined that 1 wt% MAC-SH contained 4.1 ± 0.2 mM sulfhydryl groups. Sufficient maleimide-streptavidin was covalently linked to 1 wt% MAC to allow for protein immobilization and the remainder of free sulfhydryl groups were reacted with excess maleimide-GRGDS peptide. The attachment of this peptide served to both provide attachment ligands for NSPCs and quench any previously unreacted sulfhydryls. RGD provides a native attachment ligand for differentiating NSPCs and is found in basement membrane proteins including laminin and fibronectin [1,38]. A substitute reaction with maleimide-K(FAM)GRGDS was performed to determine how much peptide was covalently attached to the material. Fluorescent measurement after purification showed that 1 wt% streptavidin-MAC-K(FAM)GRGDS contained 4.3 ± 0.3 mM peptide.
The presence of immobilized streptavidin on MAC-(GRGDS, Streptavidin) enables covalent attachment via biotinylated proteins in an aqueous solution and subsequent formation of scaffolds through photo-crosslinking of methacrylamide groups (Fig. 3A). Performing these procedures with biotinylated rIFN-γ demonstrates efficient binding to MAC-(GRGDS, Streptavidin) via admixing. Fluorescent assays revealed that all of the admixed biotin-rIFN-γ attached to the scaffold, whereas washing was able to remove 81.8 ± 2.4% of adsorbed biotin-rIFN-γ (i.e., when streptavidin was not conjugated to MAC prior to addition of biotin–rIFN-γ, Fig. 3B). This efficient coupling facilitated the synthesis of 3-D scaffolds with defined concentrations of IFN-γ.

3.3. Neural stem cell 3-D differentiation

Differentiation experiments with 3-D encapsulated NSPCs in MAC scaffolds and 100 ng/mL immobilized biotin–rIFN-γ were carried out for 8 d and compared to 100 ng/mL soluble biotin–rIFN-γ and medium without any growth factors (Figs. 4 and 5). IHC for neurons in scaffold cryosections revealed that immobilized biotin–rIFN-γ resulted in the same percentage of βIII+ neurons (72.8 ± 16.0%) as soluble biotin–rIFN-γ (71.8 ± 13.2%) after 8 d of culture (p > 0.05). The scaffold alone was insufficient to guide neuronal differentiation of encapsulated NSPCs with only 9.4 ± 2.8% of cells staining positive for the neuronal marker, βIII tubulin. Interestingly, in the presence of soluble biotin–rIFN-γ, significantly more RIP+ (oligodendrocytes) and nestin+ (stem/progenitor) cells were observed than in the presence of immobilized biotin–rIFN-γ and medium alone controls (Fig. 4, p < 0.0001). Significantly less nestin+ cells (17.8 ± 2.7%) were seen on immobilized scaffolds as compared to soluble treated scaffolds (30.8 ± 4.0%; p < 0.0001). Nestin staining can colocalize with βIII tubulin in semi-committed neuronal progenitors and immature neurons [39,40] thereby accounting for the greater than 100% of cell counts for the soluble biotin–rIFN-γ system. Considered together, these results suggest that immobilized IFN-γ results in a more neuronal committed population than soluble biotin–rIFN-γ, yet additional studies are required to probe this mechanism further. Very little GFAP positive staining was observed, which is consistent with previous studies using rat SVZ derived NSPCs [6,22,23].

4. Discussion

Photopolymerizable MAC offers a flexible platform for cells by allowing the incorporation of peculiar physicochemical cues to those found in their native niche. Using MAC scaffolds, NSPCs were shown to require a scaffold substrate with stiffness (Young’s elastic modulus) less than 1 kPa for neuronal differentiation [6]. Moreover, MAC scaffolds facilitated the covalent modification of IFN-γ to the surface (i.e., 2-D) of <1 kPa scaffolds to encourage further neuronal differentiation [23]. In this study we aimed to build upon these 2-D findings and guide neuronal differentiation of NSPCs in 3-D using a more elegant immobilization strategy as a way to achieve more efficient and specific protein coupling. This necessitated recombinant addition of biotin near the N-terminus of an IFN-γ fusion protein and covalent modification of streptavidin to MAC. By mixing NSPCs with biotin–rIFN-γ immobilized MAC hydrogels prior to photo-crosslinking, the differentiation profile was investigated in 3-D. Immobilization of growth factors is advantageous for implantation strategies where sustained and local differentiation
are promoted in defined volumes of the biomaterial scaffold. Additionally, MAC provides a photocrosslinkable platform enabling molding, layering and/or the incorporation of biochemical gradients [41,42]. While MAC is enzymatically degraded by lysosome, it did not degrade in the timeframe of the in vitro studies described herein [35].

This study demonstrates application of recombinant techniques to produce large quantities of an engineered fusion protein incorporating IFN-γ for specifying the 3-D differentiation of NSPCs into neurons. The fusion protein was produced first by designing a plasmid for *E. coli* expression that integrated a 6X histidine tag for Ni-NTA chromatography purification along with an AviTag sequence for site-specific biotinylation using the BirA enzyme (Supplementary Table 1). Despite insoluble protein expression in BL21(DE3), renaturation yielded ample milligram scale quantities of rIFN-γ fusion protein for all experiments. BirA completely biotinylated all protein as quantified by the FlowReporter Biotin Quantitation assay.

Recombinant technology offers the ability to engineer fusion proteins with nearly any desired functionalities. Fusion proteins are advantageous for protein immobilization because they provide control over protein binding and result in milligram scale (or higher depending on main culture or reactor size) quantities for downstream applications. Fusion proteins of growth factors and binding domains have been created previously to control immobilization to specific ECMs, biomaterials and cells. Fibronectin or fibronectin cell-binding domains have been incorporated in fusion proteins along with bFGF and EGF to stimulate vascularization and wound healing [43]. Collagen or collagen binding domains have also been incorporated into fusion proteins of bFGF [44], EGF [45,46], platelet derived growth factor (PDGF) [47], hepatocyte growth factor (HGF) [48] and nerve growth factor (NGF) [49] for targeted wound regeneration. Immobilization using these ECM binding mechanisms provides an equilibrium dissociation constant (*Kd*) for collagen binding on the order of 10^-7 M [50] and on the order of 10^-15 M for fibronectin [51]. The biotin–streptavidin immobilization used in this study uses a much stronger and more favorable biological association with a *Kd* on the order of 10^-12 M [52]. Utilizing this binding mechanism allowed us to controllably and stably attach rIFN-γ containing fusion protein in solution to MAC (Fig. 3).

This further facilitated the formation of pro-neurogenic NSPC-seeded 3-D scaffolds that encouraged significant neuronal differentiation after 8 d in basic medium containing no additional soluble growth factors (Figs. 4 and 5). This strategy would have been difficult and costly to perform without recombinantly produced AviTag–IFN-γ.

Synthesis of MAC-SH using Traut’s reagent was selected for ease as well as coupling efficiency. This approach resulted in greater thiolation (4.1 mM for 1 wt% MAC) than we have reported previously using thioglycolic acid and carbodiimide coupling (0.996 mM for 2 wt% MAC) [35]. The addition of sulfhydryl groups to MAC facilitated the efficient and aqueous-based chemistry of both mal- eimide-streptavidin as well as maleimide-GRGDS. Sulfhydryl conjugation is advantageous since sulfhydryl occurrence in proteins or other biomolecules is usually low (2.26% of all amino acid residues found in mammals [53]), thus bioconjugation can be highly restricted [54]. The creation of a photocrosslinkable biopolymer incorporating rIFN-γ as a pro-neuronal differentiation factor, in addition to GRGDS as an attachment ligand, allowed for the creation of neurogenic 3-D scaffolds. Using these scaffolds, with only basic medium, we were able to guide NSPCs encapsulated within to primarily differentiate into neurons (Fig. 4). Similar results were seen in 3-D scaffolds in which cells were cultured in the presence of soluble biotin–rIFN-γ. 3% tubulin positive cells were seen evenly throughout both immobilized and soluble bio- tin–rIFN-γ scaffolds (Fig. 5). These results are an improvement over previous surface (2-D) immobilization data [23] where 60 ± 4% neurons were quantified on the highest IFN-γ surface concentration of 37.5 ng/cm². Not only did this study yield greater neuronal differentiation, we also were able to achieve complete/stoi- chiometric coupling of biotin–rIFN-γ to streptavidin–MAC whereas the immobilization on 2-D surfaces used carbodiimide chemistry that had a coupling efficiency of only 8.0 ± 0.6%.

In this study, a recombinant fusion protein incorporating a growth factor was shown to couple with 3-D biomaterial scaffolding through specific, favorable and strong binding in order to specify NSPC phenotype. Previous studies with growth factor fusion proteins have almost exclusively focused on incorporation of ECM binding domains for weaker non-specific binding [43–49]. In addition to guiding NSPC differentiation, 3-D immobilization of rIFN-γ could be advantageous for controlling the spatial differentiation/activation with sustained dosing of antigen-presenting cells (dendritic cells, macrophages and B lymphocytes), hematopoetic progenitor cells and T cells [55].

5. Conclusions

In this study, we show that the synthesis of photocrosslinkable streptavidin-modified MAC along with the recombinant production of biotin-IFN-γ facilitates the creation of 3-D hydrogels with immobilized IFN-γ. These scaffolds promote the differentiation of NSPCs into neurons in 3-D in the presence of only basic medium in one week. This approach enables creation of spatially tailored biomaterials for CNS regeneration while fostering further understanding of the basic principles influencing stem cell fate.

Acknowledgments

We are grateful to partial funding from NSERC (Discovery grant to MS), Ontario Ministry of Research and Innovation postdoctoral fellowship (NDL), Fonds Québécois de la Recherche sur la Nature et les Technologies (FRQNT) and Vision Science Research Program to RWG, Ontario Neurotrauma Foundation (HK) and the Advanced Food and Materials Network (AFM-Net to M.S.). The authors would like to thank Dr. Tasneem Zahir for assistance with neural stem cell culture and assays, Dr. Ying Fang Chen for assistance with cell culture, Dr. Karen Maxwell for help and advice with recombinant protein production and Dr. Henry Krause for use of the AKTA PPLC.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.09.031.

Appendix

Figures with essential color discrimination. Figs. 2, 3 and 5 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at 10.1016/j. biomaterials.2010.09.031.

References

Dhara SK, Stice SL. Neural differentiation of human embryonic stem cells.


