Biomaterials for neural-tissue engineering — Chitosan supports the survival, migration, and differentiation of adult-derived neural stem and progenitor cells

Vanessa I. Scanga, Alex Goraltchouk, Nasser Nussaiba, MollyS. Shoichet, and Cindi M. Morshead

Abstract: Neural precursor cells (NPCs or stem and progenitor cells) are promising in transplantation strategies to treat an injury to the central nervous system, such as a spinal cord injury (SCI), because of their ability to differentiate into neurons and glia. Transplantation studies to date have met with limited success for a number of reasons, including poor cell survival. One way to encourage cell survival in injured tissue is to provide the cells with a scaffold to enhance their survival, their integration, and potentially their differentiation into appropriate cell types. Towards this end, four amine-functionalized hydrogels were screened in vitro for adult murine NPC viability, migration, and differentiation: chitosan, poly(oligoethylene oxide dimethacrylate-*co*-2-amino ethyl methacrylate), blends of poly(oligoethylene oxide dimethacrylate-*co*-2-amino ethyl methacrylate), and poly(vinyl alcohol), and poly(glycerol dimethacrylate-*co*-2-amino ethyl methacrylate). The greatest cell viability was found on chitosan at all times examined, Chitosan had the greatest surface amine content and the lowest equilibrium water content, which likely contributed to the greater NPC viability observed over three weeks in culture. Only chitosan supported survival of multipotent stem cells and the differentiation of the progenitors into neurons, astrocytes, and oligodendrocytes. Plating intact NPC colonies revealed greater cell migration on chitosan relative to the other hydrogels. Importantly, long term cultures on chitosan showed no significant difference in total cell counts over time, suggesting no net cell growth. Together, these findings reveal chitosan as a promising material for the delivery of adult NPC cell-based therapies.

Key words: biomaterials, chitosan, hydrogels, stem cells, cell viability.

Résumé : Les cellules neurales précurseurs (CNP, cellules souches et progéniteurs) sont très prometteuses dans les stratégies de transplantation pour le traitement de blessures au système nerveux central, tel un traumatisme au cordon médullaire (TCM), en raison de leur habilité à différencier les neurones des cellules gliales. Jusqu'à maintenant, les études de transplantation n'ont eu que des succès mitigés en raison de divers facteurs, dont le faible taux de survie des cellules. Une façon d'augmenter ce taux de survie dans les tissus traumatisés est de fournir des cellules comportant un échafaudage permettant d'augmenter leur survie, leur intégration et éventuellement leur différentiation dans les divers types de cellules. À cette fin, quatre hydrogels portant des amines fonctionnalisées, le chitosane, le poly(diméthacrylate de l'oxyde d'oligoéthylène-*co*-2-aminométhacrylate d'éthyle), des mélanges de poly(diméthacrylate de l'oxyde d'oligoéthylène-*co*-2-aminométhacrylate d'éthyle) et d'alcool polyvinylique et du poly(diméthacrylate de glycérol-*co*-2-aminométhacrylate d'éthyle) ont été soumis à une évaluation in vitro, pour la migration, la différentiation et viabilité de cellules neurales précurseurs de murine adulte. Dans tous les cas, la viabilité la plus grande des cellules a été observée avec le chitosane. Le chitosane comporte le degré le plus élevé d'amine de surface et la quantité la plus faible d'eau en équilibre, ce qui a vraisemblable-

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ment contribué à la plus grande viabilité des cellules neurales précurseurs sur une période de trois semaines dans les cultures. Seul le chitosane permet de supporter la survie de cellules souches à plusieurs composantes et de différencier les progéniteurs en neurones, astrocytes et oligodendrocytes. L'examen de plaques de colonies intactes de cellules neurales précurseurs met en évidence que la migration de ces cellules sur le chitosane est beaucoup plus rapide que sur les autres hydrogels. Les cultures à long terme sur le chitosane ne présentent pas de différences significatives dans les comptages totaux de cellules en fonction du temps; cette observation est importante puisqu'elle suggère qu'il ne supporte aucune croissance nette de cellules. Toutes ces observations mettent en évidence le fait que le chitosane est un matériau plein de promesses pour conduire à des thérapies basées sur les cellules neurales précurseurs adultes.

Mots-clés : biomatériaux, chitosane, hydrogels, cellules souches, viabilité des cellules.

[Traduit par la Rédaction]

Introduction

Traumatic injury to the adult mammalian spinal cord causes irrevocable damage, producing an environment that inhibits regrowth of damaged axons.[1–4] The initial damage to the cord is compounded by a prolonged secondary cascade of injury including the disappearance of growth-promoting cues, the appearance of growth-inhibiting factors, apoptosis, and the formation of a glial scar. This series of post-injury cascades leads to the formation of a cystic cavity, axonal degeneration, and ultimately an inability of the adult cord to self-repair.[5,6] Regeneration of severed central-nervous-system (CNS) axons has been reported for both cellular bridges[7–9] and biomaterial implants;[10–13] however, functional recovery from these single application treatments has been limited. The complicated pathophysiology resulting from spinal cord injury (SCI) likely necessitates a combination strategy to promote axonal repair and regeneration. Neurons, astrocytes, and oligodendrocytes comprise the primary cell types of the CNS and because these cells die at the site of injury after SCI, cell replacement therapies will undoubtedly be key to combination strategies aimed at repairing the damaged spinal cord.

The therapeutic potential of CNS stem cells has received considerable attention.[14–27] The observation that stem-cell populations can be isolated from along the developing and adult neuroaxis[28,29] has led to the development of a number of cell-based therapies to treat SCI. In the context of developing regenerative-medicine strategies, the use of adult-derived populations is a significant step forward as it affords an approach that is independent of ethical concerns. Adult neural stem cells are maintained throughout the life of the animal in both the brain and spinal cord and have the capacity to self-renew and differentiate into astrocytes, oligodendrocytes, and neurons.[28,30–33] Neural stem cells can be isolated and propagated in vitro using a well-described colony-forming assay whereby individual stem cells proliferate in the presence of epidermal growth factor (EGF) and (or) fibroblast growth factor (FGF2) to form free-floating colonies of cells termed "neurospheres". Individual neurospheres can be dissociated into single cells and replated in the presence of these growth factors to form new neurospheres thereby resulting in an expansion of neural stem-cell-derived cell populations, and demonstrating the cardinal stem cell property of self-renewal. Individual neurospheres are comprised of a mixed population of stem and progenitor cells[31] and are referred to collectively as neural precursor cells (NPCs). Upon exposure to differentiation conditions, neurosphere-derived cells can differentiate into all of the neural phenotypes comprising the CNS (neurons, astrocytes, and oligodendrocytes), thereby illustrating their multipotentiality. The ability to proliferate and differentiate into CNS-specific cell types makes NPCs promising candidates for cell replacement strategies to replace cells lost after CNS injury.

The extracellular matrix during development influences cell adhesion, growth, differentiation, and motility[34] while at the same time contributing to tissue strength.[35] The regenerative ability in the damaged adult CNS is limited in large part due to the release of inhibitory matrix proteins and a breakdown in the structural scaffolding provided by the extracellular matrix to neurons, astrocytes, and oligodendrocytes.[36,37] Accordingly, bridging strategies provide attractive models of repair following damage to the nervous system and have included the use of nerve guides,[38,39] hydrogel scaffolds,[40] and tissue and cell transplants such as fetal tissue, Schwann cells, olfactory ensheathing glia, neural stem cells, and progenitor cells[27,41–45] as well as macrophages.[46] The partnership of some of these bridging strategies with the administration of neurotrophic[47–49] or survival[50–53] factors have clearly demonstrated the powerful effects of combination therapy in the treatment of this debilitating injury. However, these studies have shown only modest functional improvement. The extent of intrinsic cell renewal following the local delivery of mitogenic agents such as EGF and FGF2 to stimulate endogenous precursor cells[47] has also been insufficient in promoting significant recovery following SCI. Additionally, the poor survival rate of cells transplanted directly into the site of injury following SCI[54] reflects the need to provide a better environment for cell survival. Work by Karimi-Abdolrezaee et al.[14] has shown that combining NPC transplantation and the delivery of growth factors via an osmotic mini-pump leads to a dramatic increase in cell survival and differentiation and, moreover, leads to measurable functional recovery. Hence, we propose that a nerve guidance channel that is biodegradable, biocompatible, supportive of NPC survival and differentiation, and amenable to the incorporation of a drug delivery system will promote axon regeneration following SCI. Towards this end, we set out to identify the microenvironment, and specifically the matrix material, that met the specific criteria of NPC viability, migration, and differentiation.

Four polymeric biomaterials were screened using neurosphere-derived NPCs isolated from the forebrain of adult mice. These biomaterials were required to be biode-

gradable, amine-functionalized (to promote cell adhesion), non-cytotoxic, and capable of being processed into a nerve guidance channel. The following biomaterials were compared in terms of NPC viability, cell migration, and differentiation: (*i*) chitosan, a naturally-derived polysaccharide; (*ii*) poly(oligoethylene oxide dimethacrylate-*co*-2-amino ethyl methacrylate), P(PEG-*co*-AEMA); (*iii*) blends of P(PEG-*co*-AEMA) and poly(vinyl alcohol) (PVA), P(PEG-*co*-AEMA)–PVA; and (*iv*) poly(glycerol dimethacrylate-*co*-2-amino ethyl methacrylate), P(GDMA-*co*-AEMA). Chitosan was studied because it has already demonstrated some promise in neural-tissue engineering[55] and PEG-AEMA based polymers were synthesized with the understanding that PEG would be biocompatible[56] while AEMA would provide amine functional groups for enhanced cell adhesion.

Materials and methods

Materials

All reagents were purchased from Sigma-Aldrich and used as received unless otherwise stated. Poly(ethylene glycol) dimethacrylate (PEGDMA, molecular weight: 400 Da) and poly(vinyl alcohol) (PVA, molecular weight: 6000 Da, hydrolyzed to 80%) were purchased from PolySciences Inc. (Warrington, PA, USA). The MicroBCA Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). Deionized water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA, USA) and used at 18 MΩ resistance.

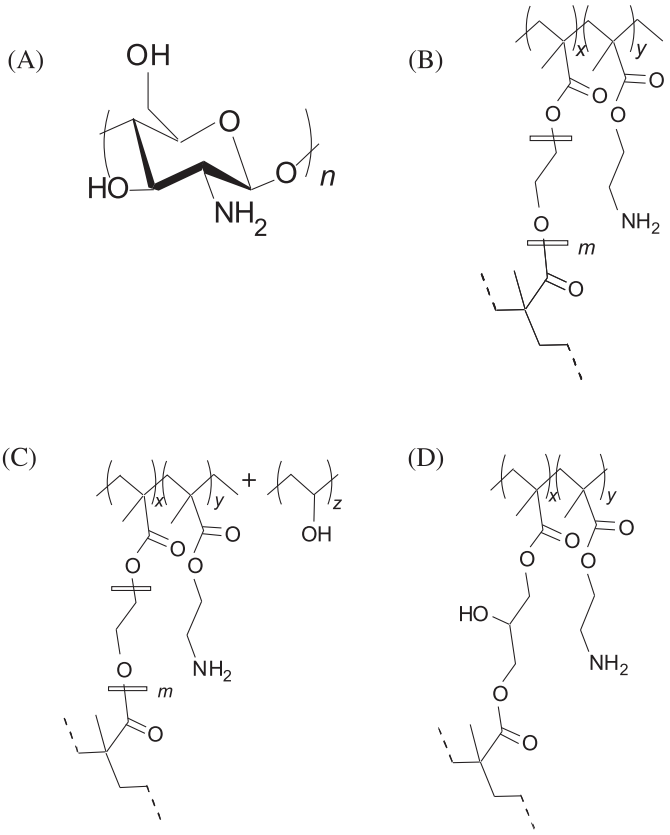
Preparation of chitosan films

Deacetylated chitosan films were prepared from chitosan (NovaMatrix, Norway, M_w: approximately 150 000–400 000 g/mol) by two successive alkaline hydrolysis steps with 40 wt% NaOH solution at 100 °C for 1 h. The hydrolyzed flakes were washed to neutrality with water and lyophilized using the Thermo Savant ModulyoD freeze dryer at –50 °C and 1.5 mbar pressure. Following lyophilization, 5 g of deacetylated chitosan flakes were added to 166.7 mL of 2 wt% acetic acid in water solution (2/3 working solution). A stock neutralization solution was prepared by mixing 30 mL of ammonia with 70 mL of water and 900 mL of ethanol. Five to six g of the chitosan working solution was poured into a 10 cm Petri dish and allowed to stand uncovered for 24 h. The air-dried films were then washed twice with 20 mL of neutralization solution for 1 h each time. The neutralized films were then washed with PBS and allowed to dry overnight (Fig. 1A).

Synthesis of PEGDMA-co-AEMA films

One g of PEGDMA was added to 20 mL of water in a 30 mL vial. The solution was vortexed and 20 μL of concentrated phosphoric acid was added to keep the pH below 4.0. One gram of 2-amino ethyl methacrylate (AEMA) was added to the solution and allowed to completely dissolve. Stock solutions of 10 wt% ammonium persulfate (APS) and 10 wt% sodium metabisulfite (SMBS) in water were prepared. After complete dissolution of the monomers, 100 μL of each of the APS and SMBS stock solutions were added, and the solution was vortexed and transferred to a 10 cm Petri dish for polymerization at 37 °C for 24 h (Fig. 1B).

Fig. 1. Four amine-functionalized polymer films were synthesized to assay neural precursor cell - matrix interaction: (A) chitosan, (B) poly(oligoethylene oxide dimethacrylate-*co*-2-amino ethyl methacrylate [P(PEGDMA-*co*-AEMA)], (C) physically blended poly(vinyl alcohol) and P(PEGDMA-*co*-AEMA) [P(PEGDMA-*co*-AEMA)–PVA], and (D) poly(glycerol dimethacrylate-*co*-2-amine ethyl methacrylate) [P(GDMA-*co*-AEMA)].



The disks were washed 3 times with PBS for 1 h each time and then washed in PBS overnight prior to use. We did not expect any adverse response due to residual APS–SMBS based on previously published results.[55]

Synthesis of PEGDMA-co-AEMA-blend–PVA films

The identical method as described for the synthesis of PEGDMA-*co*-AEMA films was followed with the dissolution of the PEGDMA in 20 mL of a 5 wt% solution of PVA (6000 Da) in water instead of water alone in the first step (Fig. 1C).

Preparation of GDMA-co-AEMA films

One g of glycerol dimethacrylate (GDMA) and 20 μL of concentrated phosphoric acid were dissolved in a 1:1 (*v/v*) mixture of water and acetone. After the pH was lowered to pH < 4.0, to minimize rearrangement of the AEMA monomer via the acyl migration mechanism, 1 g of AEMA was added to the solution. Upon complete dissolution of all the monomers, 100 μL of the APS and SMBS stock solutions were added, and the solution was vortexed and transferred to a 10 cm glass Petri dish covered with a glass slide. The dish with the polymerizing mixture was placed in an oven at 83 °C for 40 min. Soon after gelation, but prior to the

change in turbidity, the films were cooled to room temperature and 10 mL of water added (Fig. 1D).

Analysis of polymer films

Polymer films were analyzed for equilibrium water content (EWC) according to eq. [1].

$$[1] \qquad EWC = \frac{m_w - m_d}{m_d}$$

Wet mass (m_w) was measured after allowing 5 mm disks of each material to swell in water for 2 weeks. Dry mass (m_d) was measured after freeze-drying. The surface chemical composition was analyzed by X-ray photoelectron spectroscopy (XPS) in survey mode and for specific elements (C, N, O) using the Mg Kα X-ray photoelectron spectrometer [Leybold (SPECS) Max 200].

Isolation and culturing neural precursor cells

Neural precursor cells (NPCs) were isolated from the subependyma lining the lateral ventricles in the forebrain of adult CD1 mice (Charles River) as previously described.[57] Bulk cultures were maintained at clonal densities (10 cell/μL) in 1% penicillin–streptomycin serum-free medium (P/S-SFM) supplemented with EGF (20 ng/mL), FGF2 (10 ng/mL), and heparin (7.32 ng/mL) according to Tropepe et al. All supplements were purchased from Sigma-Aldrich (Oakville, ON, CA). The cultures were passaged weekly[57] to a maximum of three weeks.

NPC plating and immunocytochemistry

Prior to cell plating, the polymer biomaterial films (8.6 mm^2) were disinfected with 100% ethanol for 5 min, followed immediately by three serial washes in standard phosphate-buffered saline. The films were placed at 37 °C for 2–3 h prior to cell plating. Cell populations were plated at a density of 3×10^4 single cells/film by slowly pipetting cells across the surface of the material. At 24 h, the plates were examined to ensure that there was no clumping of cells over the film surface. Cell migration studies involved plating 10 whole neurospheres onto the surfaces of each of the biomaterials tested and comparing to Matrigel™ controls. Cell counts were conducted in five non-overlapping areas of the polymer films as observed in 10× fields of view of each sample area. An in-vitro experimental paradigm was established to analyze the viability of cells within a defined time course. Cell-seeded films were exposed to standard neurosphere-forming media described above followed by the addition of fetal bovine serum (+FBS) on day 2 post-plating to induce differentiation. Cultures were maintained for 2, 7, or 19 days after the addition of FBS and cell-seeded discs were analyzed at 4 (2 days with EFH + 2 days with FBS), 9 (2 + 7), and 21 (2 + 19) days post-seeding. Day-21 samples received new media every 7 days. Sample counts of Hoechst-positive cells were made at 10× magnification per field of view. To compare cell counts over time and in the center and periphery of the film surfaces, 50% of the total film area was defined as the center area and 50% defined as the perimeter area. In all cases, 6–10 individual wells containing single material discs ($n \geq 3$ independent trials) were analysed per group, per trial. In a separate set of experiments, cell viability on chitosan was analysed and compared to Matrigel™ usng a PicoGreen assay (Invitrogen) on d0, 2, 4, 9, and 21 post-plating ($n = 3$ discs/trial from 3 independent trials). NPCs were plated in growth factor conditions for 2 days followed by 1% FBS for an additional 2, 7, or 19 days, similar to the Hoechst counting study described above. Cells were lysed off the films, and along with d0 samples, lysates were stored at –80 °C. Lysates were processed according to the manufacturer's instructions and DNA content was measured using a fluorescent microplate reader (excitation 480 nm, emission 520 nm). The concentration of dsDNA per lysate sample was converted to an estimation of total viable cells (6.05ρg DNA / diploid mammalian cell).

To determine whether neural stem cells persisted on chitosan following exposure to differentiation conditions, cells plated on chitosan or Matrigel™ for 9 d were lifted from chitosan surfaces by washing three times with standard PBS followed by a 5 min incubation in 500 μL of 0.25% trypsin-EDTA at 37 °C. Each well sample was triturated five times using a fire-polished glass pipette to dislodge the cells from chitosan surfaces. Cells were centrifuged for 5 min at 1500 rpm, and the supernatant was replaced with a 10 mg / 15 μL solution of trypsin inhibitor (Roche) followed by centrifugation (5 min at 1500 rpm). The supernatant was replaced with 500 μL SFM including EGF, FGF2, and heparin. Cells were replated onto non-adherent 24-well plates and neurosphere formation was assayed 7–9 days later. Recovered neurospheres were dissociated and passaged in standard neurosphere culture conditions and the presence of new neurospheres was assayed after 7 days. Neurosphere-derived cells were plated in 1% fetal bovine serum to examine their differentiation profile using standard immunocytochemistry as described below.

Immunocytochemistry was conducted as reported.[57,58] Briefly, single cells derived from dissociated neurospheres were plated onto the surfaces of biomaterials in 1% P/S-SFM containing FBS in the presence of growth factors following the culture conditions described above. Two, 7, and 19 days after plating the cell-seeded materials were fixed with 4% paraformaldehyde for 20 min. Cells were stained with antibodies to glial fibrillary acidic protein (GFAP, 1:400 rabbit polyclonal, Chemicon), nestin (1:1000 mouse monoclonal, Chemicon), βIII tubulin (1:500 mouse monoclonal, Sigma-Aldrich) and O_4 (1:75 mouse monoclonal, Chemicon). Hoechst 33258 (0.725 μL/mL, Sigma-Aldrich) was used to visualize the nuclei of plated cells to estimate the total number of cells as described above. To determine the percentages of differentiated cell types, the number of immunolabeled cells was counted and expressed as a percentage of the total number of Hoechst positive cells from 5 nonoverlapping fields of view per well (≥3 wells/group/antibody, $n \geq 3$ independent trials). Appropriate secondary antibodies (FITC or Alexa goat anti-rabbit, goat anti-mouse; FITC, or TRITC (1:200) from Jackson, Alexa (1:400) antibodies from Molecular Probes) were used. Secondary-only controls were prepared in the same manner as cell-plated biomaterials with 10% normal goat serum (Jackson) replacing primary antibodies. Cells were visualized on an Olympus IX70 fluorescent microscope with Olympus Microsuite Software.

Statistical analysis

Student *t* test was performed for statistical analysis with 95% confidence using Microsoft Excel statistical software.

Results

Cell counts are greater on chitosan compared with P(PEGDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA)–PVA blends and P(GDMA-*co*-AEMA)

A key limitation with cell transplantation is the poor rate of cell survival following implantation. For improved therapeutic efficacy following nervous system damage, an increased rate of cell survival is key to success. To determine which of the four biomaterials examined (chitosan, P(PEGDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA)–PVA blend, or P(GDMA-*co*-AEMA)) best supports cell adhesion and viability of neurosphere-derived NPCs, the nuclear marker Hoechst was used to compare cell counts at 4 and 9 d post-plating. Cells showed a relatively even distribution on each material surface at 24 h post-plating; however this changed over time, depending on the material. As shown in Fig. 2A, at 4 d there were significantly more NPCs on chitosan at $2.33 \pm 0.053 \times 10^3$ (comparable to Matrigel™ at $2.00 \pm 0.01 \times 10^3$) than on the other hydrogel polymers studied, with the fewest number of NPCs found on P(GDMA-*co*-AEMA), followed by P(PEGDMA-*co*-AEMA). and P(PEGDMA-*co*-AEMA)–PVA. The low number of NPCs present on the synthetic polymer surfaces between d4 and d9 reflects either poorly maintained cell adhesion or poor cell survival. The lack of cell survival was further highlighted by the appearance of smaller nuclei,[59] which is indicative of poor cell viability, at both d4 and d9 on P(GDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA), and P(PEGDMA-*co*-AEMA)–PVA (Figs. 2B and 2C). Both chitosan and Matrigel™ controls showed greater total numbers of cells surviving on d9 relative to d4, suggesting proliferation of NPCs between these two time points. This proliferation of NPCs observed on chitosan between d4 and d9 prompted the examination of longer survival times in culture because the continued proliferation of cells would be an undesirable attribute of the cell-material interaction. Cell counts on chitosan and Matrigel™ plated for 4, 9, and 21 d in vitro revealed that by d21 the total number of Hoechst-positive cells was not significantly different from the d4 counts on either matrix ($p = 0.17$ for d4 compared to d21 on Matrigel™ and $p = 0.21$ for d4 compared to d21 on chitosan). A PicoGreen assay was also performed at 2, 4, 9, and 21 days post-plating to determine the number of viable cells over time and relative to day 0. The assay employs a fluorochrome that selectively binds double-stranded DNA and can be used to extrapolate the numbers of viable cells per sample. We determined the numbers of viable cells at day 2 (prior to the addition of FBS) as a baseline to reflect the amount of cell death that occurred following initial plating. We observed a decrease in the numbers of cells on both chitosan and Matrigel™ on day 2 (Fig. 2D); however, between d4 and d9, we observed a 1.6- and 1.7-fold increase in the numbers of viable cells on chitosan and Matrigel™, respectively, and similar to what was observed with the Hoechst counts (Fig. 2A). As with the Hoechst analysis, the numbers of viable cells on day 21 was not significantly different from the numbers present on d4. Overall, these data suggest that chitosan supports greater NPC survival and not uncontrolled cell proliferation. While chitosan and Matrigel™ demonstrated similar cell survival, Matrigel™ is derived from a mouse sarcoma and consists of a multitude of proteins and factors that would not be acceptable for implantation. Moreover, unlike chitosan, Matrigel™ cannot be easily processed into a nerve guidance channel. Taken together, these data suggest that chitosan is the most promising of the materials tested in terms of promoting cell adhesion and survival.

XPS and EWC analyses

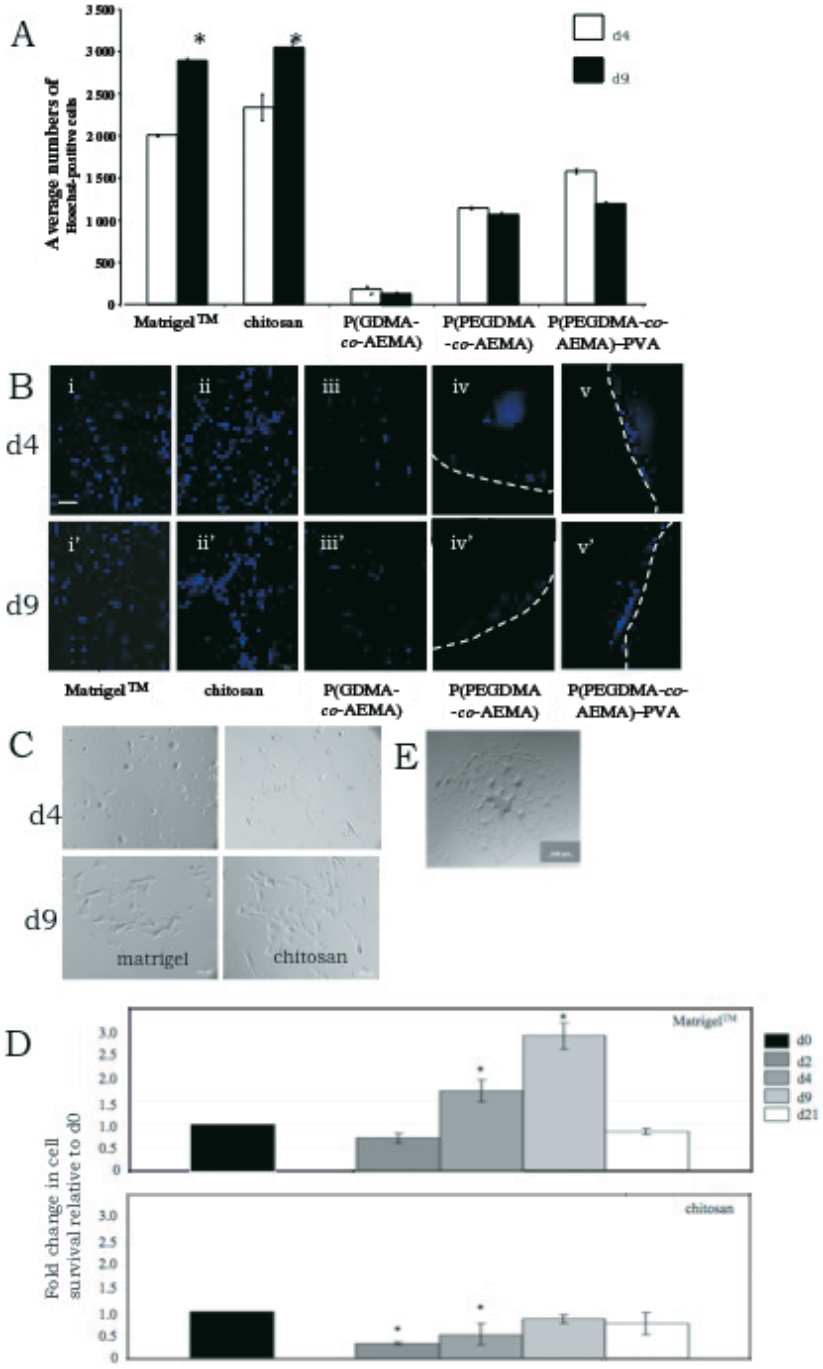
To better understand the NPC–matrix interaction, chitosan, P(GDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA), and P(PEGDMA-*co*-AEMA)–PVA were analyzed by X-ray photoelectron spectroscopy (XPS) for surface composition and bulk equilibrium water content (EWC). As shown in Table 1, of the four biomaterials tested, chitosan had the highest nitrogen content (2.75 ± 0.45) and thus the highest amine surface concentration, reflecting that it is approximately $99.2 \pm 0.5\%$ deacetylated chitin.[60] Interestingly, chitosan also had the lowest EWC. Our finding that NPC counts are greatest on chitosan is in agreement with Freier et al. who suggested that the higher amine content in chitosan results in increased cell viability relative to films with lower cationic ammonium charge, likely because of non-specific interactions with the negatively charged cell membranes.[60] P(GDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA), and P(PEGDMA-*co*-AEMA)–PVA have significantly greater water content and thus were more hydrophilic than chitosan, reflecting the significant water capacity of PEG and AEMA hydrogels. PEG has been used repeatedly to limit cell adhesion[61] and protein adsorption;[62] thus, despite copolymerization with AEMA to introduce amine functional groups, the high water content likely contributed to the limited cell adhesion and viability observed.

Neural precursor colony adhesion

Based on the XPS and EWC analysis performed, we hypothesized that a greater initial adhesion of NPCs to the chitosan surface relative to P(GDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA), and P(PEGDMA-*co*-AEMA)–PVA may be a reason for the higher cell counts on chitosan. To test this, we plated 10 intact colonies of NPCs (neurospheres) on each biomaterial surface using identical culture conditions to those used for single cell plating, and examined the number of neurospheres that adhered to each surface at 9 days after cell plating. We observed a greater number of neurospheres adhering to the surface of chitosan relative to the hydrogel polymers (80% on chitosan versus 20%–30% on the other polymers tested). These differences support the hypothesis that the greater amine surface concentration and decreased water content of chitosan, relative to the synthetic hydrogels tested, led to greater cell adhesion and thus greater survival on chitosan.

The plating of whole neurospheres allowed us to look at cell migration on the surfaces of the polymer matrices. A prominent feature of NPCs and their progeny, both in vivo[63–65] and in vitro,[26] is their ability to migrate. We directly tested cell migration on all four biomaterial films

Fig. 2. (A) Cell viability was analyzed at d4 and d9 counting Hoechst-positive nuclei. Data are shown as mean ± SEM, $n = 3$ independent trials of 6–10 films. All synthetic materials have significantly reduced numbers of cells present at 4 and 9 days relative to Matrigel™ controls. * indicates significant difference relative to day 4 ($p < 0.05$) (B) At d4 (top row) and d9 (bottom row) most cells on P(GDMA-*co*-AEMA) (iii, iii′), P(PEG-*co*-AEMA) (iv, iv′), and P(PEG-*co*-AEMA)–PVA (v, v′) were notably smaller and regionalized to the perimeter of the discs compared to chitosan (ii, ii′) and Matrigel™ controls (i, i′). Dotted lines indicate the edge of the disk where cells were exclusively observed on P(PEG-*co*-AEMA) and P(PEG-*co*-AEMA)–PVA. (C) Bright phase images of cells on chitosan and Matrigel™ showing the morphology of the adherent cells on d4 and d9 post plating single cells. (D) Cell viability analyzed on Matrigel™ and chitosan at 2, 4, 9, and 21 days in culture using the PicoGreen assay and showing the fold change in the numbers of viable cells relative to the numbers of seeded cells on d0. Data represents means ± sem. * = significant difference relative to d0 ($p < 0.05$). (E) Whole neurosphere on chitosan at d9 showing adhesion and migration of cells. Scale bar = 100 μm.



compared to Matrigel™ controls by examining the diameter of adherent neurospheres (Fig. 2E). When intact neurospheres of equal diameter (100 μm) were plated on day 0 and the diameter of the adherent colony was examined on d4, the cells migrated significantly more on chitosan relative to the other polymeric surfaces tested (chitosan: 2.13 ±

Table 1. X-ray photoelectron spectroscopy and water equilibrium content analysis.

Material	Nitrogen surface concentration (%)	Equilibrium water content
Chitosan	2.75 ± 0.45	8.01 ± 1.37
P(GDMA-*co*-AEMA)	0.35 ± 0.05	56.74 ± 1.81
P(PEGDMA-*co*-AEMA)	1.65 ± 0.15	50.71 ± 3.51
P(PEGDMA-*co*-AEMA)–PVA	0.5 ± 0.20	54.68 ± 1.87

0.75 mm; P(GDMA-*co*-AEMA): 0.37 ± 0.18 mm; P(PEGDMA-*co*-AEMA): 0.49 ± 0.14 mm, and P(PEGDMA-*co*-AEMA)–PVA): 0.51 ± 0.36 mm). Hence, chitosan supports cell adhesion and migration to a greater extent than the other biomaterials tested herein, making it a promising material for cell delivery.

NPC differentiation

Neural precursor cells have the potential to differentiate into astrocytes, oligodendrocytes, and neurons in vitro in the presence of serum on Matrigel™.[29,34] To test the differentiation capacity of NPCs on the candidate matrices, we examined the differentiation profile using immunohistochemistry at various times post-plating. Single NPCs plated onto each biomaterial (and Matrigel™ controls) were immunostained for glial fibrillary acidic protein (GFAP) expression to assess for astrocyte formation; βIII tubulin for neurons; and O_4 for oligodendrocytes (Figs. 3A and 3B). Strikingly, chitosan was the only candidate material of the four analyzed that supported NPC differentiation regardless of the time in culture (d4, d9, or d21). Moreover, chitosan supported the differentiation of all three cell types (astrocytes, neurons, and oligodendrocytes), similar to what is observed on Matrigel™ (Fig. 3A); however, the relative percentages of the differentiated phenotypes varied between the two substrates. Notably, neurons were observed on both chitosan and Matrigel™ at d9 and an increase in the percentage of neurons was observed on chitosan over time; oligodendrocytes were present at each time point and the percentage was not statistically different on chitosan over time; however the percentage of oligodendrocytes decreased on Matrigel™, and astrocytes were present at each time point on both chitosan and Matrigel™ and comprised the major cell type at all time points. While it is still not known which cell type, or combination of cell types, is best suited for enhancing neural regeneration, the fact that all cell types can be formed on chitosan suggests that it may be advantageous for developing cell transplantation strategies using nerve guidance channels following nervous system damage.

Neural stem cells persist on chitosan

The neurosphere-derived cells seeded onto the matrices were a mixed population of stem and progenitor cells. Having observed the multipotentiality of the progenitor cells on chitosan, we questioned whether neural stem cells (i.e., the neurosphere-forming cells) also persisted on the matrix. We assayed for the survival of stem cells by lifting the cells off the matrix 9 days post-plating and placing the cells in culture conditions that permit neurosphere formation. We examined the cells in terms of their ability to re-form neurospheres and for the expression of nestin (an intermediate filament protein that is expressed by virtually 100% of neurosphere-derived NPCs prior to differentiation and continues to be expressed following differentiation in vitro). We observed nestin expression following differentiation on chitosan at d4, d9, and d21 post-plating (Fig. 3), suggesting that undifferentiated precursors were maintained on chitosan. Notably, there was significant overlap in the GFAP- and nestin-expressing populations (ie. single cells expressed both markers) which may reflect an immature phenotype of astrocytes in culture.[66,67] Importantly, we isolated neurosphere-forming cells from chitosan cultures in comparable numbers to those observed on Matrigel™. Similarly, the recovered neurospheres could be passaged and gave rise to neurons, astrocytes, and oligodendrocytes following differentiation. Hence, chitosan supports the differentiation of all three neural cell types in addition to the survival of multipotent stem cells.

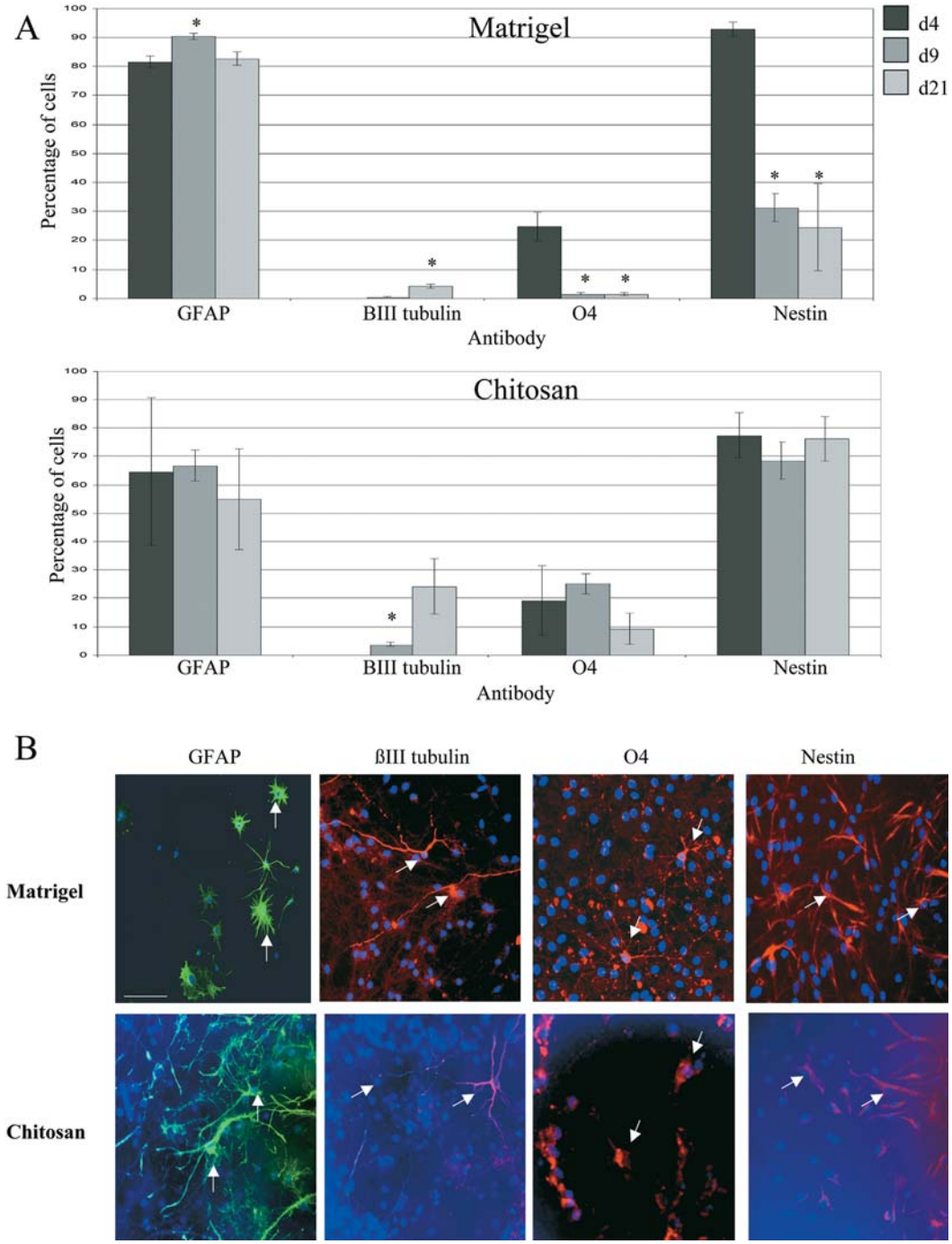
Discussion

It has been shown that extrinsic factors originating from both diffusible factors[68–71] and cellular interactions[72–74] can regulate the proliferation and differentiation of cells. To our knowledge, this report is the first to show that cell viability, migration, and differentiation of adult murine NPCs differ over time on distinct amine-functionalized polymer surfaces. Moreover, the study identifies adult-derived NPCs and chitosan matrices as promising candidates for surgical implantation strategies aimed at restoring neurological deficits following injury to the nervous system.

Recently, Young et al.[74] reported that chitosan and poly(vinylidene fluoride) (PVDF) substrates could support the proliferation and differentiation of embryonic-derived cortical precursors. Interestingly, their observations using an embryonic-derived starting population of cells generated notable differences from our study when comparing the behaviour of cells plated on chitosan. Our starting population of single adult-derived cells was capable of differentiating into the three neural cell types on chitosan (Fig. 3), which differs from what was found using embryonic cortical precursor populations where single cells were inhibited from proliferating and differentiating on chitosan. This is a good example of the importance of considering both the material and the starting population of cells when designing strategies for tissue repair.

An important criterion in biomaterial selection in this study was the cell–biomaterial interaction. Irrespective of time in culture, NPC counts were significantly greater on chitosan than on the other synthetic hydrogels analyzed, demonstrating the importance of surface composition and water content which is consistent with the observations of others.[27,75–77] P(GDMA-*co*-AEMA), P(PEGDMA-*co*-AEMA), and P(PEGDMA-*co*-AEMA)–PVA hydrogels were either covalently or physically cross-linked to form highly water-swellable polymer networks. The high water content of these polymeric hydrogels minimized protein adsorption and cell adhesion[78,79] reflecting the high water content associated with PEG.[80] To facilitate cell adhesion, amine-functionalized AEMA was copolymerized with GDMA and PEGDMA; however, this proved insufficient to overcome the low protein- and cell-adhesion properties associated with PEG. In-

Fig. 3. (A) The quantification of single-plated neural precursor cells for GFAP (astrocytes), neurons (ßIII tubulin), oligodendrocytes (O4) and Nestin (undifferentiated cells) on Matrigel™ controls and chitosan on d4, d9, and d21. * indicates significant difference relative to day 4 ($p < 0.05$). (B) Immunohistochemistry of neural precursor cells plated on Matrigel™ and chitosan at d9 showing individual cells expressing GFAP (green), ßIII tubulin (red), O4 (red), and Nestin (red) as indicated by arrows. Nuclei are Hoechst-positive (blue). Immunohistochemistry captured on chitosan exhibits an increased fluorescent background relative to Matrigel™. Scale bars = 100 μm.



terestingly, there was a progressive increase in cell viability and adhesion with decreasing water content in the polymer films studied and it was water content, not amine functionalization, that had the overriding effect on cell adhesion and thus viability. The low equilibrium water content, coupled with the amine functional groups present on chitosan, rendered chitosan cell-adhesive. Chitosan has also been shown to be biocompatible and to degrade to non-cytotoxic products,[60,81] making it desirable for further investigation.

Adult NPCs are proliferative cells that migrate along a well-defined pathway to the olfactory bulb where they differentiate into neurons in vivo.[58,63,82–85] Proliferation, migration, and differentiation are also well-established characteristics of NPCs in vitro.[86] The cell behaviour observed

was dependent on the cell-matrix interaction and accordingly varied significantly between the matrices. Cell proliferation was observed on chitosan and control matrices between d4 and d9 and this coincided with an increase in the total numbers of cells in the center of the film. This mitogenic effect on chitosan surfaces has also been seen with human fibroblast and keratinocyte populations in vitro[87] and is thought to be the result of the highly deacetylated chitosan potentiating the effect of growth factors present in culture. Importantly, the total numbers of cells on d21 did not continue to increase, thereby reducing the likelihood of uncontrolled cell growth in vivo following implantation strategies after CNS damage. The ability of chitosan to promote the differentiation of all cell types (neurons, astrocytes, and oligodendrocytes) may be advantageous because its therapeutic potential as a delivery vehicle could be maximized for CNS injury repair and is attractive for developing regenerative medicine strategies. Importantly, chitosan is amenable to the incorporation of a drug delivery system that will further facilitate the development of specific cell types over time. Taken together, these features make chitosan a promising material for future development in the treatment of nervous system injury. More broadly, our findings provide the necessary framework for combining cellular therapeutics and implantation strategies in tissue engineering repair strategies of the damaged nervous system.

Conclusions

Of four biomaterials screened, chitosan supported the greatest survival, migration, and multipotent differentiation of adult-derived neural precursors, making it a promising candidate material for cell-biomaterial transplantation studies in CNS applications. Importantly, chitosan supported the survival of multipotent, self-renewing stem cells without uncontrolled proliferation. Taken together, these findings provide the necessary framework for combining cellular therapeutics and implantation strategies to repair the damaged CNS.

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