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Heparin crosslinked chitosan microspheres for the delivery of neural stem cells and growth factors for central nervous system repair

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ABSTRACT

An effective paradigm for transplanting large numbers of neural stem cells after central nervous system (CNS) injury has yet to be established. Biomaterial scaffolds have shown promise in cell transplantation and in regenerative medicine, but improved scaffolds are needed. In this study we designed and optimized multifunctional and biocompatible chitosan-based films and microspheres for the delivery of neural stem cells and growth factors for CNS injuries. The chitosan microspheres were fabricated by coaxial airflow techniques, with the sphere size controlled by varying the syringe needle gauge and the airflow rate. When applying a coaxial airflow at 30 standard cubic feet per hour, ~300 μm diameter spheres were reproducibly generated that were physically stable yet susceptible to enzymatic degradation. Heparin was covalently crosslinked to the chitosan scaffolds using genipin, which bound fibroblast growth factor-2 (FGF-2) with high affinity while retaining its biological activity. At 1 μg ml^{-1} approximately 80% of the FGF-2 bound to the scaffold. A neural stem cell line, GFP + RG3.6 derived from embryonic rat cortex, was used to evaluate cytocompatibility, attachment and survival on the crosslinked chitosan–heparin complex surfaces. The MTT assay and microscopic analysis revealed that the scaffold containing tethered FGF-2 was superior in sustaining survival and growth of neural stem cells compared to standard culture conditions. Altogether, our results demonstrate that this multifunctional scaffold possesses good cytocompatibility and can be used as a growth factor delivery vehicle while supporting neural stem cell attachment and survival.

1. Introduction

The brain is arguably the most difficult organ to repair after an injury due to the complexity of the central nervous system (CNS) and its limited capacity to regenerate on its own. Neurons do not undergo mitosis and endogenous neural stem cells are unable to replace the quantity of neurons lost after a typical injury. One week post-injury a glial scar forms, which creates an inhibitory environment eliminating the possibility of axonal regeneration [1–3]. Exogenous neural stem cell transplants for brain injury have garnered interest, but effective paradigms for transplanting these cells have yet to be established. Injecting neural precursors directly into the penumbra of an injury has yielded limited success. For example Harting et al. [4] showed that less than 2% of the donor cells engraft and survive in the host brain for 1 year. Transplantation of cells alone may not be enough to overcome the harsh environment, loss of supportive matrix and other problems resulting from brain injury. It follows logically that these cells will benefit from transplantation upon a scaffold [5–8]. One possible reason that the survival rate of transplanted cells is low is that the cystic cavity formed by the injury creates a harsh, non-permissive environment that lacks nutrients, survival factors and, most importantly, a habitable substrate. A scaffold would serve as a structural and functional support for the cells.

Brain injuries are not uniform in shape or size; therefore a scaffold that is injectable and will mold to the injured tissue will be necessary. Hydrogels would fit this criteria; however, cells, particularly neurons, do not extend their processes or neurites efficiently through three-dimensional (3-D) matrices [9–14]. The neurite outgrowth is best observed on 2-D rigid structures. Microspheres contain such a 2-D rigid structure on their surface, as opposed to the 3-D soft structure of hydrogels. Growth cones of neurons pull on their neurites, requiring tension to maintain or initiate neurite extension, which is greater on microspheres than on hydrogels. Another disadvantage using hydrogels is that their biodegradation is hard to control [15,16]. In addition, microspheres can also be fabricated to deliver specific growth and trophic factors to aid cellular engraftment and survival of the transplanted stem cells [17].

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Many biomaterials have been explored in neural tissue engineering applications, including natural materials such as alginate, collagen and Matrigel, or synthetic polymers such as poly(lactic acid) and poly(glycolic acid). Synthetic polymers, while versatile and amenable to adjusting their mechanical or degradation properties, may be inappropriate for brain tissue engineering applications. Firstly, they can leach cytotoxic substances, and, when degraded, their by-products are often acidic, adversely affecting the local brain tissue and increasing inflammation [18]. Furthermore, these polymers are not similar to natural proteins within the body. In particular, they lack the functional groups that natural polysaccharides contain. This results in a lack of cell recognition signals, decreasing the potential for cell adhesion (if desired) and increasing the likelihood that a fibrous scar will form around the scaffold. Natural polymers mimic the native ECM proteins, thus favoring cell interaction or immobilization. However, because these materials are naturally derived, they can be expensive, not readily available and impractical for large scale processing. Another problem may be their immunogenicity, as they are extracted from other animals or plants. Collagen is a common polymer explored in tissue engineering applications that requires some processing to acquire. Although collagen type IV is typically found in the brain, collagen type I is more widely used because it is less expensive. A disadvantage with collagen use in the brain is its potential for foreign body reaction [19]. Matrigel is non-homogeneous protein matrix derived from tumor cells. While cells may interact favorably with this material, it is not a clinically applicable material due to its undefined nature. Other potentially suitable materials, such as hyaluronic acid and fibronectin, are very costly and are difficult to chemically modify.

Chitin is the second most abundant natural polysaccharide in the world next to cellulose. Chitosan is derived by alkaline deacetylation of chitin, which yields repeating units of glucosamine and N-acetylglucosamine in its polymer chains. The percent deacetylation of chitosan governs its properties. The number of acetyl or amine groups at the C2 position determines its mechanical properties, degradation and biocompatibility. Degradation can be easily reduced by crosslinking the polymer. Slowing the degradation time of the scaffold for in vivo applications would enable the biodegradation rate to parallel the rate of new tissue formation [20,21]. Unlike many other polymers, chitosan requires mild processing conditions, dissolving in water with a low acidity (pH < 6.3). Chitosan elicits a minimum foreign body reaction, having been used as an anti-microbial agent [22] and for drug delivery [23], wound healing [20,24] and tissue engineering [25–28]. The US Food and Drug Administration has approved its use in multiple applications. Chitosan has also shown potential in neural tissue engineering applications [7,8,29–33]. Several groups have explored the potential of chitosan as a microcarrier for immunosuppressants [34,35], cancer drugs [36–38] and growth factors [8,29,39]. Guo et al. [40] showed an increase in stem cell survival when transplanted within a chitosan matrix containing Nogo-66 receptor protein in spinal cord injuries.

The cationic nature of chitosan allows interaction with anionic glycosaminoglycans (GAGs) such as hyaluronan or heparin. Heparin is a well-known anticoagulant and plays a role in angiogenesis [41,42], which could aid in revascularizing the damaged cortex. Heparin also has been demonstrated to reduce inflammation [43,44]; thus it may be able to decrease inflammation in the brain and promote engraftment. Another important property of heparin is its high affinity for growth factors such as fibroblast growth factors, hepatocyte growth factor (HGF), platelet-derived growth factor, vascular endothelial growth factor (VEGF) and bone morphogenic protein-6 (BMP-6) [45]. Heparin binds these growth factors and maintains their stability by preventing their thermal degradation [46]. Fibroblast growth factor-2 (FGF-2) is a known survival factor for many types of stem cells, including neural stem cells. During development, FGF-2 promotes neural stem cell self-renewal and maintains the stem cells in a primitive state. It has also been shown to increase the proliferation of the endogenous neural precursors in the subventricular zone following traumatic brain injury, as well as providing neurogenic effects [47,48]. FGF-2 also increases neural stem cell migration and neuronal differentiation [49,50]. Importantly, it binds with high affinity to heparin. The stability and controlled release of FGF-2 over a designated timeframe will help keep the neural stem cells primitive, surviving and proliferating after transplantation.

One of the advantages of using chitosan as the bulk material for a fabricated scaffold is its similarity to natural GAG, which allows easy modification of its side chain groups. Heparin is a naturally occurring highly sulfated GAG that can bind ionically to the amine groups on chitosan via its sulfate and carboxylate side chains. These growth factor–heparin–chitosan complexes can be exploited to produce a biocompatible drug delivery mechanism. Temperature, pH, ions, fluid flow and cytokines may all play a role in the removal of ionically bound heparin as well as degradation of these complexes. Genipin, a plant-derived crosslinking agent, possesses similar mechanical crosslinking properties to the chemical glutaraldehyde, but without its corrosive, cytotoxic and carcinogenic side effects. Mi et al. [51] transplanted chitosan-only microspheres and those crosslinked with glutaraldeyhyde or genipin into skeletal muscles. The genipin crosslinked microspheres elicited less inflammation than the glutaraldehyde crosslinked chitosan. Genipin has been used to covalently bind heparin to chitosan to produce a stable scaffold complex that is ideal for clinical use [52,53]. Genipin has also been suggested to be neurogenic and anti-inflammatory [54–58].

Although chitosan-based microspheres have been widely used in drug delivery and tissue engineering applications, there have been no reports of genipin crosslinked chitosan–heparin complex microspheres for the delivery of neural stem cells and growth factors for CNS repair. In this study, we designed and optimized chitosan-based microspheres as a cellular and growth factor delivery vehicle for nervous tissue regenerative applications. The studies that we performed were designed to test the hypothesis that chitosan–heparin complexes can be used as an effective scaffold for FGF-2 binding and neural stem cell growth and survival.

2. Materials and methods

2.1. Reagents

Chitosan (low molecular weight, ~50 kDa, 75–85% deacetylation), heparin sodium salt from bovine intestinal mucosa and MTT (3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium) were purchased from Sigma (St Louis, MO). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lysozyme was purchased from MP Biomedicals (Solon, OH). rh-FGF-2 and FGF-2 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Peprotech (Rocky Hill, NJ).

2.2. Preparation of chitosan microspheres

Chitosan powder (0.5, 1.0, 1.5, 2.0 or 2.5 g) was dispersed in 50 ml of water containing 2.0 vol.% acetic acid to create 1, 2, 3, 4 and 5% chitosan solutions. The chitosan solution was mechanically stirred at 700 rpm until completely dissolved. The resulting solution was collected and centrifuged at 2000 rpm for 10 min. Subsequently, the supernatant was collected and the remaining impurities that pelleted were discarded. Chitosan microspheres were formed using a coaxial airflow technique [59]. Briefly, the