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Physical chitosan microhydrogels as scaffolds for spinal cord injury restoration and axon regeneration

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ABSTRACT

Recovery from traumatic spinal cord injury (SCI) usually fails due to a cascade of cellular and molecular events that compromise neural tissue reconstitution by giving rise to glial scarring and cavity formation. We designed a scaffold material for SCI treatment containing only chitosan and water as fragmented physical hydrogel suspension (Chitosan-FPHS), with defined degree of acetylation (DA), polymer concentration, and mean fragment size. Implantation of Chitosan-FPHS alone into rat spinal cord immediately after a bilateral dorsal hemisection promoted reconstitution of spinal tissue and vasculature, and diminished fibrous glial scarring: with astrocyte processes primarily oriented towards the lesion, the border between lesion site and intact tissue became permissive for regrowth of numerous axons into, and for some even beyond the lesion site. Growing axons were myelinated or ensheathed by endogenous Schwann cells that migrated into the lesion site and whose survival was prolonged. Interestingly, Chitosan-FPHS also modulated the inflammatory response, and we suggest that this might contribute to tissue repair. Finally, this structural remodeling was associated with significant, long-lasting gain in locomotor function recovery. Because it effectively induces neural tissue repair, Chitosan-FPHS biomaterial may be a promising new approach to treat SCI, and a suitable substrate to combine with other strategies.

1. Introduction

Traumatic injuries of the spinal cord (SCI) can lead to life-long loss of sensation and voluntary motor functions. Although severed adult neurons of the mammalian central nervous system (CNS) can initially survive, regrowth of their axons through the lesion site ultimately fails due to a cascade of cellular and molecular events leaving the affected neural tissue in a permanently altered, regeneration-inhibited state (for review, see [1,2]). A further complication is that injury-associated vascular damage inevitably includes hypoxia, hemorrhage and edema that accelerate necrosis of damaged neural tissue. The associated breakdown of the blood-spinal cord barrier favors infiltration of blood-derived monocytes/macrophages that, together with activated microglia, spread inflammation beyond the initial lesion site, contributing to secondary expansion of the lesion. The tissue cavities that develop because of necrosis become walled off by the astrocytic scar, creating a mechanical, and chemical barrier to axon growth due to expression of inhibitory molecules such as CSPG, as well as semaphorins, and matrix-metalloproteinases. Axon regrowth is further inhibited by central myelin degradation products, namely MAG (myelin associated glycoprotein), Omgp (oligodendrocyte myelin glycoprotein), and Nogo, and also by low levels of neurotrophic factors (for review, see [3,4]).

To overcome these hurdles, implantable biomaterials have recently gained interest in the treatment of SCI. To be effective, these biomaterials must first bridge the gap created by the lesion in the spinal tissue by forming an extracellular matrix replacement that supports anchoring of cells and promotes axon growth. Secondly, they should reduce tissue cavitation and glial scarring, allowing for efficient neo-vascularization. Thirdly, and importantly,

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they need to participate in modulating the long-term inflammatory response [5,6].

Scaffold materials to treat SCI can be entirely synthetic polymers, such as PLA (poly-L-lactic acid and polylactide copolymers; [7], PHEMA/MMA (poly-(2-hydroxyethyl methacrylate-co-methyl methacrylate]; [8], or PEG (polyethylene glycol; [9]; or can be derivatives of naturally occurring polymers, like fibrin [10], alginate [11], or chitosan [12,13]. The biodegradability of these components can vary from non-degradable to rapidly degrading biomaterials. Some materials can be electrospun to form solid fibers; most come in the form of hydrogels (containing >70% water) that can either be implanted, or form a gel *in situ* from an aqueous solution. These scaffolds may also be used to deliver growth-promoting molecules, and/or can contain grafted cells favoring neural tissue regeneration and myelination of regrowing axons (for review, see [14]).

Chitosan, used in our novel biomaterial for SCI treatment, is prepared by partial *N*-deacetylation of chitin, a natural polysaccharide obtained from the exoskeleton of crustaceans, the cell wall of fungi, and or from squid pens. The physicochemical properties of chitosan can be modulated, in particular through the variation and control of its average degree of acetylation (*DA*). Chitosan-based materials and derivatives have received increased attention in tissue engineering because of their unique and appealing properties such as cytocompatibility, biodegradability, and nontoxicity. They have been extensively evaluated for regeneration of epithelial and soft tissues [15], and their beneficial characteristics include preservation of cellular phenotypes, activity enhancement of bound bioactive factors, as well as the stimulation of synthesis of tissue-specific extracellular matrix [16,17]. In addition, it has antitumor activity, as well as analgesic, hemostatic, anticholesterolemic, antimicrobial, and antioxidant effects (for review, [13,15]).

In treating SCI, chitosan has been successfully employed in the form of collagen-filled or stem cell-seeded preformed tubes designed to bridge the tissue gap created by transection of rat spinal cord [12,18–21]. However, these studies did not evaluate the therapeutic potential of the chitosan biomaterial alone. The impact of the hydrogel implant itself on scar formation, inflammation, and vascularization was not analyzed, with one exception [12] reporting that chitosan did not provoke a higher than normal inflammatory reaction. Efficacy of the chitosan-biomaterial alone could provide major advantages as a therapeutic agent, avoiding potential safety issues encountered by cotreatments with cell based therapies, and making it easier for its large scale production for clinical testing. In the present study, we evaluated the reaction of lesioned spinal cord tissue to a pure chitosan-based implant in form of a newly designed formulation as a Fragmented Physical Hydrogel Suspension (Chitosan-FPHS) with well defined physico-chemical characteristics, i.e. molecular weight, acetylation degree, polymer concentration, and range of fragment size.

2. Materials & methods

2.1. Preparation of Chitosan-FPHS

Chitosan powder from squid pens (Mahtani Chitosan, Veraval, India; batch index 114) was dissolved in acetic acid aqueous solution, pressure-filtered through successively narrower Millipore filters (3.0, 1.2, and 0.45 μm pore size), and re-precipitated by mixing with concentrated ammonia solution. Precipitates were washed in deionized water until neutral pH was achieved, and finally freeze-dried before further use. Number average and weight average molar masses (M_n and M_w) were determined by size exclusion chromatography coupled with refractive index measurements and multiple light scattering (Wyatt Dawn EOS multi-angles operated at 690 nm), according to our previous work [22]. The degree of acetylation (*DA*) was analyzed by ^{1}H-NMR spectroscopy on chitosan (10 mg/mL) dissolved in D_2O acidified with 5 μL/mL of concentrated HCl according to the method of Hirai [23]. Reacetylation of chitosan was performed by dropwise addition of stoichiometric amounts of acetic anhydride (>99% purity, Sigma) to a 1% w/v chitosan hydro-alcoholic solution, then letting the reaction continue overnight before re-precipitating the polymer with concentrated ammonia, again followed by washing steps and lyophilisation (for details see [16], and refs. therein).

A 2.5% w/w aqueous solution of filtered chitosan, obtained by addition of a stoichiometrically determined amount of acetic acid to protonate amine groups of chitosan, was poured into a 35 mm Petri dish. Chitosan hydrogel formation was achieved by placing the Petri dish over a source of ammonia vapors (generated with an aqueous ammonia solution, 1 M) for 15 h, and the resulting chitosan monolithic gel was washed in deionized water until a neutral pH was obtained in the washing bath. This final hydrogel only contains chitosan and water at a concentration close to that of the initial solution [22,24]. Evaluation of the viscoelastic properties and mechanical behavior of such bulk hydrogels was done according to our previous work [22,24]. The storage modulus at equilibrium corresponding to the plateau value of G′ measured at low angular frequency (~10^{-2} rad/s) of our final hydrogel was close to 1300 Pa (for more details, see [24]). The gel was then fragmented by blending in deionized water with an Ultra Turrax® (IKA) homogenizer (10% [w/w] of gel in deionized water, rotation speed 11,000 rpm; 4 × 20 s; Fig. 1A). Blending time and rotation speed were chosen so that the fragmented suspension had a median fragment size of about 20 μm. The suspension was autoclaved at 121 °C for 20 min, and finally kept at room temperature. The chitosan used in this study had a *DA* of 4% ±0.5%, a M_w of about 550 ± 50 kg/mol, a dispersity $Đ$ (equal to M_w/M_n) of 1.5 ± 0.3 [22]. Such high molecular masses enable the preparation of hydrogels at low concentration (Cp > 1%w/w), here 2.5%. The initial suspension was centrifuged (Sigma 3K30 at 13,000 rpm, 30s) and the pellet was collected. The apparent viscosity of the Chitosan-FPHS paste was determined as shown in Fig. 1B. Hydrogels prepared from high DA chitosan (38% ±2%; obtained by reacetylation of low DA chitosan) but the same fragment size as above (20 μm), as well as a hydrogel with low DA (4%, Fig. 1A) but much larger fragment size (~150 μm, Fig. 1A) were also tested. The Chitosan-FPHS preparation was based on the procedure described in Nothias et al. [25].

2.2. Animals

Adult female Wistar rats (230–250 g; total number of animals: 84; Janvier, France) were used throughout this study, housed in the animal facilities at the IBPS institute. All experimental procedures and animal care were in accordance with 2010/63/UE European directive and French decree 2013-118, and approved by the Institutional Animal Care and Ethics Committee at the UPMC (#1514.01). Before and after surgical interventions animals were provided food and water *ad libitum*. During surgery, and until they awoke from anesthesia, animals were placed on a heating pad to maintain body temperature. To relieve post-operative pain, rats received intraperitoneal injections of Buprenorphine (0.3 mg/mL, Axience, France) twice a day during the first week post-lesion, and intramuscular injections of gentamycin (Pangram 4%, Virbac, France) to prevent urinary infections. Until restoration of normal micturition, bladders were manually emptied twice a day, and the health state of operated rats was monitored by regular weight.