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Chitosan-based hydrogel implants enriched with calcium ions intended for peripheral nervous tissue regeneration

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ABSTRACT

A new method for fabrication of chitosan-based hydrogel implants intended for peripheral nervous tissue regeneration was developed. The method is based on an electrodeposition phenomenon from a solution of chitosan and organic acid. In order to increase the mechanical strength of the implant, the solution was enriched with hydroxyapatite. Hydroxyapatite served as a source of calcium ions too. The influence of the concentration of the polymer and the additive on chemical, mechanical as well as biological properties of the obtained implant was evaluated. The study showed great dependence of the initial solution composition mainly on the physicochemical properties of the resulting structure. Basic *in vitro* cytotoxic and pro-inflammatory assays showed biocompatibility of manufactured implants, therefore, animal experimentations may be considered.

1. Introduction

Each year, medical statistics note over 600,000 patients with peripheral nerve injuries (PNIs) in Europe and in the United States (Jonsson et al., 2013; Liu et al., 2012b). PNIs may or may not cause disruptions of axons, with subsequent loss of sensory function and motor weakness. In a case when the nerve injury leaves a gap between transected nerve stumps, axons go on regenerating supported by associated glial cells (Navarro, Vivó, & Valero-Cabré, 2007). However, many axons are misdirected to inappropriate targets (de Ruiter, Spinner, Verhaagen, & Malessy, 2014; Hamilton et al., 2011) and a neuroma is formed (Macias, Lehman, Sanger, & Riley, 1998). This condition results in a functional impairment of the nerve fiber.

The development of an ideal technique to repair transected peripheral nerves is up to date a challenge for medical doctors as well as biomaterial engineers. The damaged nerves with small gaps (smaller than 8 mm) are treated by an end-to-end coaptation of impaired nerve stumps. In a case of PNIs in which the direct suture of the cut ends can result in huge tension, an approach based on inserting a bridging implant is used. The standard clinical procedure for treating the transected nerves with a gap exceeding 8 mm is grafting a pure sensory nerve (*e.g.*, sural nerve) (Kuffler, 2014). However, such approach has some disadvantages. First of all, the sensory nerve graft is not suitable for promoting the regeneration within mixed sensory and motor nerves because the outgrowth of motor axons is promoted by different neurotrophic factors than the one of sensory axons (Brenner et al., 2006; Nichols et al., 2004). Moreover, the regeneration of mixed nerves can be inhibited by inappropriate diameters of the sensory nerve graft (de Ruiter et al., 2014). The diameter of mixed nerves is usually bigger than the one of sensory grafts. This mismatch results in the creation of a toxic environment. On the other hand, the use of mixed nerve grafts is limited by ethical considerations (Kuffler, 2014).

Due to the above-mentioned concerns, development of novel implants in the form of nerve guidance conduits (NGCs) for peripheral nerve regeneration has received recently a great deal of attention (Pfister et al., 2011). There are many biological as well as physicochemical requirements for materials intended to serve as constituents of NGCs (Ramburrun et al., 2014). They should

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be characterized by appropriate biological, biomechanical, and physicochemical properties similar to the ones of healthy nerves (Faroni, Mobasseri, Kingham, & Reid, 2015). Materials which are currently under investigation to serve as NGCs can be divided into two main groups: synthetic devices and natural (resorbable) devices. These of synthetic origin can be additionally resorbable or non-resorbable. In the literature, many excellent reviews which describe synthetic as well as natural origin NGCs can be found (Kehoe, Zhang, & Boyd, 2012; Nectow, Marra, & Kaplan, 2012; Siemionow, Bozkurt, & Zor, 2010).

When choosing an appropriate structural component of NGCs, special attention is put on natural polymers, especially chitosan (Liu, Ma, Mao, & Gao, 2011). Chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine. It is derived from chitin, the second after cellulose most abundant polysaccharide found in nature. Chitosan reveals favorable biological properties including biodegradability, non-toxicity, and remarkable adhesion to living tissues. It has an excellent neuroglial cell affinity and exhibits almost no cytotoxicity on the growth of Schwann cells (Yuan, Zhang, Yang, Wang, & Gu, 2004). Moreover, chitosan has been shown to attract extracellular matrix molecules (*e.g.*, laminin, fibronectin, and collagen IV) that are responsible for promoting nervous cells to adhere, migrate, and differentiate (Nomura et al., 2008).

Recently, enrichment of the structure of NGCs with signaling molecules enhancing the outgrowth of axons has been studied. One of factors that have been identified as a crucial for appropriate regeneration of nerves is calcium. However, its potential to constitute the structure of implants has not been studied yet. It has been shown that calcium ions play a crucial role in the development of both single nervous cells and functional connections between them (Rosenberg & Spitzer, 2011). Calcium-mediated signaling pathways take part in the specification of neuronal subtype, dendritic growth and arborization, and specification of neurotransmitter subtype. Moreover, calcium signaling is responsible for the regulation of both the rate of axonal outgrowth and the directional navigation of a growth cone to the axon's target. However, there seems to be an optimal level that allows promoting the successive increase in the outgrowth of nerves (Henley & Poo, 2004).

Taking the above-mentioned needs into account, the aim of the presented investigations was to develop a new method of obtaining nerve guidance implants in the form of chitosan-based hydrogel. A straight-forward method of manufacturing based on an electrodeposition phenomenon from a solution of chitosan and organic acid was elaborated. In order to increase mechanical strength of the implant, the solution was enriched with hydroxyapatite. Hydroxyapatite served as a source of calcium ions too. The chemical and mechanical properties of obtained implants were characterized through mechanical testing, microscopy (*i.e.*, SEM), and spectroscopy (*i.e.*, FTIR, XPS). Biocompatibility, one of the most important factors when medical applications are considered, was primarily assessed through cytotoxicity and pro-inflammatory testing.

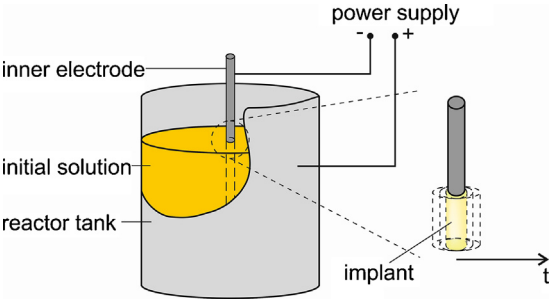

Fig. 1. Schematic diagram of chitosan-based hydrogel implants manufacturing process.

Table 1
Initial composition of solutions used for chitosan-based hydrogel implants fabrication.

Abbreviation	Chitosan (g)	Hydroxyapatite (g)
CH 1	0.4	0.05
CH 2	0.4	0.1
CH 3	0.6	0.05
CH 4	0.6	0.1

2. Experimental

2.1. Materials

Chitosan (CH, CAS48165, high viscosity > 400 mPa s, 1% in acetic acid (20 °C)) and hydroxyapatite (HA, CAS677418 nanopowder, <200 nm particle size (BET)) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lactic acid (LA, CAS69775) was acquired from Fluka (Buchs, Switzerland).

2.2. Implant manufacturing

Method of implants fabrication developed in our laboratory is schematically presented in Fig. 1 and was described earlier in a patent application (P. 406608, 2013). Briefly, chitosan solution was prepared by dissolving 0.4 or 0.6 g of chitosan in 100 mL of 0.3 M lactic acid 3 wt/vol% LA. Then, 0.05 or 0.1 g of hydroxyapatite was added to the solution. The obtained solution was stirred (under slow rotations) until complete dissolution for 24 h. Then, 30 mL of the solution was put into a reactor tank of a specially designed reactor. The reactor is composed of two stainless steel (316L) electrodes, inner and outer, and is closed from bottom and top by two flanges of insulating material. The inner electrode has a diameter of 2 mm and the outer one 30 mm. The reaction process, that is present in the reactor, is based on an electrodeposition phenomenon. The electrodeposition was conducted for 10 min at 25 °C and the voltage was set at 12 V. The process was conducted for solutions with four different initial contents of CH and HA (Table 1). The applied initial amount of the solution allowed fabricating a 65 mm long implant. After this time, the obtained implants were removed from the inner electrode and subjected to chemical, physical, and biological analysis of their properties.

2.3. Structural studies

Mechanical testing was performed on samples just after fabrication. Samples for SEM, FTIR, and XPS analysis were taken immediately after preparation and frozen to the temperature −25 °C. Then, they were lyophilized at temperature 6 °C for 5 h on a Christ Alpha 2–4 apparatus.

2.3.1. Implant morphology – SEM

To study a cross-section of manufactured implants as well as their surface morphology, the obtained samples were frozen in liquid nitrogen and transversally cut with the use of scalpel before being lyophilized. Scanning electron microscopy pictures of gold-coated cross-sections of the implants were taken with a Hitachi TM-1000 machine.

2.3.2. Implant structure characterization – FTIR

The FTIR spectra were obtained using a THERMO SCIENTIFIC Nicolet™ iS™10 FT – IR apparatus in the range of 4500–650 cm^{-1}. The device was equipped with a monolithic diamond ATR crystal.