JOURNAL OF THE MECHANICAL BEHAVIOR OF BIOMEDICAL MATERIALS 60 (2016) 256–266

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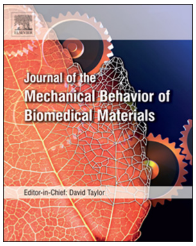

Research Paper

# Tubular electrodeposition of chitosan–carbon nanotube implants enriched with calcium ions

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ARTICLE INFO

*Article history:*
Received 9 October 2015
Received in revised form
30 January 2016
Accepted 5 February 2016
Available online 11 February 2016

*Keywords:*
Electrodeposition
Chitosan
Carbon nanotubes
Hydroxyapatite
Nerve regeneration

## ABSTRACT

A new approach for obtaining chitosan–carbon nanotube implants enriched with calcium ions in the form of tubular hydrogels is fostered. The intended application of the hydrogels is tissue engineering, especially peripheral nervous tissue regeneration. The fabrication method, based on an electrodeposition phenomenon, shows significant advantages over current solutions as implants can now be obtained rapidly at any required dimensions. Thus, it may open a new avenue to treat patients with peripheral nerve injuries. Either single walled or multiwalled carbon nanotubes enhance the mechanical properties of the tubular hydrogels. The controlled presence of calcium ions, sourced from hydroxyapatite, is also expected to augment the regenerative response. Because *in vitro* cytotoxic assays on mouse cell lines (L929 fibroblasts and mHippoE-18 hippocampal cells) as well as pro-inflammatory tests on THP-1XBlue™ cells show that the manufactured implants are biocompatible, we next intend to evaluate their immune- and nervous-safety on an animal model.

## 1. Introduction

An increasing need for improving the life quality of patients suffering from peripheral nerve injuries (PNIs) has inspired scientists to seek for alternative approaches to existing solutions. Nowadays, the standard clinical procedures for treating PNIs, that are based on autogenic or allogenic grafting, are being replaced by biofunctionalized polymeric implants called nerve guidance conduits (NGCs) (Faroni et al., 2015). The newest concept is focused on creating a structure with properties mimicking key peripheral nervous system developmental mechanisms, and thus enhancing regeneration of transected

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http://dx.doi.org/10.1016/j.jmbbm.2016.02.012
1751-6161/

peripheral nerves (Gu et al., 2014; Chiono and Tonda-Turo, 2015).

The recent findings show that an ideal implant for peripheral nerve tissue regeneration (PNTR) should both physically and biochemically resemble properties of a peripheral nerve extracellular matrix. First of all, the NGC should be in the form of a semipermeable hydrogel membrane that incorporates haptotactic, chemotactic, and mechanical cues to spatiotemporally navigate the nerve cell outgrowth as well as ensures the avoidance of inappropriate connections (Struzyna et al., 2014). Recent studies have shown that nervous cells are also sensitive to topographical cues (Stoll et al., 2014). In addition, the production of implant should ensure its sterility and flexibility facilitating its further surgical handling (Yang et al., 2010). Despite a wide variety of off-the-shelf available NGCs, their use is still problematic. The main obstacles are the structural properties of implants that should be strictly tailored to mechanical requirements (*i.e.*, ability to withstand the compression stress from the surrounding tissues and to bend without kinking), target degradation rate (*i.e.*, degradation rate adjusted to the nerve regeneration rate), and permeability characteristics (*i.e.*, appropriate pore sizes and porosity) (Chiono and Tonda-Turo, 2015). The length of the conduit should be sufficient to connect the nerve gap without generating tension. Another limitation is the standardized dimensions of prefabricated conduits that do not meet requirements of multifariousness of human peripheral nerves. Moreover, biomaterial-based implants are often not suitable for long-term storage.

Due to the above-mentioned concerns, it seems to be reasonable to seek for an alternative method to produce nerve guidance conduits. Currently, one of the most often studied components of polymeric implants is chitosan. This naturally derived polymer is chosen due to its broad spectrum of favorable properties: biocompatibility, biodegradability, and low toxicity (Shukla et al., 2013). Moreover, the structure of chitosan is similar to the one of glycosaminoglycans which are natural constituents of peripheral nerve tissue. In spite of being promising in treatment of PNIs (Gnavi et al., 2013), chitosan-based NGCs show very poor mechanical strength. Moreover, in order to be more advantageous in treating of PNIs they need to be enriched in neuronal growth signaling molecules.

Recently, the addition of carbon nanotubes (CNs) to biomaterials became a research area of great interest, since their presence significantly improves their chemical as well as physical properties (Hopley et al., 2014). The ability of CNs to promote growth and neurite elongation makes them particularly appealing for nerve tissue reconstruction (Fabbro et al., 2013). Therefore, both single-walled carbon nanotubes (SWCNs) and multi-walled carbon nanotubes (MWCNs) have been widely tested as constituents of NGCs, especially in combination with chitosan (Huang et al., 2011; Gupta et al., 2015). Interestingly, the literature shows that MWCNs show higher biocompatibility than SWCNs (Schrand et al., 2007).

One of biomaterials that have positive effects on the axonal growth is hydroxyapatite (HA). It has been shown that HA guides neuronal growth at the target direction through activating Netrin-1 signaling pathway mainly because of the presence of calcium ions (Liu et al., 2012). Chitosan has been combined with HA to form an implant intended for nerve reconstruction (Itoh et al., 2003). Despite enhancing nerve regeneration, the method of tubular implant fabrication is complicated and the products easily collapse upon bending or compression forces.

Taking the above-mentioned advances and newly emerged needs, an effort has been made in order to elaborate a straightforward method for producing chitosan-based implants in the form of tubular hydrogels. The intended application of these structures is tissue engineering, in particular peripheral nerve tissue reconstruction. The fabrication method is based on an electrodeposition phenomenon and was described earlier (Nawrotek et al., 2016). In order to improve chemical as well as mechanical properties of implants, carbon nanotubes were incorporated in their structure. Two types of CNs were chosen: single-walled and multi-walled carbon nanotubes. The equipment of implants with neuronal guidance cues was ensured by adding hydroxyapatite to the reaction solution. The physicochemical properties of obtained structures were studied through mechanical testing, microscopy (*i.e.*, scanning electron microscopy (SEM)), and spectroscopy (*i.e.*, Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS)). Biocompatibility, one of the most important factors when tissue engineering applications are considered, was primarily assessed through cytotoxicity assays using mouse L929 fibroblasts and mHippoE-18 hippocampal cells and by testing the pro-inflammatory potential upon contacting the implants with THP-1XBlue™ cells.

## 2. Experimental section

### 2.1. Materials

Chitosan (CH, high viscosity > 400 mPa s, 1% in acetic acid (20 °C)), hydroxyapatite (HA, nanopowder, < 200 nm particle size (BET)), single-walled carbon nanotubes (SWCN), and multi-walled carbon nanotubes (MWCN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lactic acid (LA) was acquired from Fluka (Buchs, Switzerland).

### 2.2. Implant manufacturing

A novel fabrication method of chitosan–carbon nanotube implants enriched with calcium ions is based on our patent application (P. 406608; Tylman et al., 2013). Briefly, 100 or 10 mg of SWCN or MWCN were ultrasonically dispersed in 100 mL of 0.3 M lactic acid for 2 h in a water bath. Then, 0.4 of chitosan and 0.05 or 0.1 g of hydroxyapatite were added to the solution. The obtained solution was stirred (under slow rotations) until complete dissolution for 24 h. Next, 30 mL of the solution was poured into a tank of a specially designed reactor (Fig. 1). The reactor is composed of two stainless steel (316 L) electrodes, inner (ø 2 mm) and outer (inner diameter, ø 30 mm), and is limited from bottom and top by two flanges of insulating material. The reaction process, that occurs in the reactor, is based on an electrodeposition phenomenon conducted for 10 min at 25 °C and with the initial voltage set at 12 V. The process was performed for solutions with four