



Enhancement of nerve regeneration along a chitosan nanofiber mesh tube on which electrically polarized β -tricalcium phosphate particles are immobilized

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ABSTRACT

The ability of β -tricalcium phosphate (β -TCP) particles to store electric charge was confirmed by thermally stimulated depolarization current measurement as well as surface potential measurement. The efficacy of stored electrical charge on β -TCP particles in enhancing nerve regeneration was evaluated. Bridge grafting was performed into sciatic nerve defects in Wistar rats with the following tubes: chitosan mesh tubes; chitosan mesh tubes on which β -TCP particles with or without electrical polarization treatment had been immobilized (polarized and non-polarized tubes, respectively). As a control, isografts were used. Both motor and sensory nerve function as well as electrophysiological recovery progressed with time in each group. Immunofluorescence revealed rapider nerve regeneration in the polarized tube group compared with the non-polarized tube group. The axon density and axon area in the polarized tube group were significantly greater than those in the chitosan mesh tube and non-polarized group, and showed no significant differences from the control group. These results suggest that the stored charge on electrically polarized β -TCP particles immobilized on chitosan mesh tubes may enhance nerve regeneration to the same extent as isografting.

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1. Introduction

The repair of peripheral nerve lesions has been attempted by bridging with a scaffold, which includes implantation and tubulization techniques. At present, autogenous nerve grafting is the most successful method for nerve repair. A wide variety of biomaterials, however, have been suggested for the production of artificial devices for nerve repair, and biodegradable scaffolds may be an alternative [1]. Chitosan has a number of beneficial biological properties; enhancing wound healing, suppressing bacterial growth and easy fabrication into various shapes, such as films, sponges and fibers. Chitosan mesh tubes with a deacetylation rate (DAC) of 93% have been developed [2]. Given the molecular characteristics as well as sufficient mechanical strength, these tubes may be substitutes for autogenous nerve grafts. The functional and histological results, however, have revealed that nerve regeneration was delayed in chitosan mesh tube grafts relative to autografts [2]. This result indicates that the nerve conduit must offer additional advantages, such as enhanced growth of Schwann cells or the growth cone and adsorption of bioactive molecules on the tube surface.

It is reported that negatively charged surfaces of electrically polarized hydroxyapatite (HA) ceramics enhance bone bonding when transplanted into canine femora [3]. To explain this phenomenon it was hypothesized that the polarizing charge could induce the orientation of OH^- ions in the HA structure [4,5]. Dehydroxylation during the measurement of electrical conductivity in air led to an increase in conductivity due to an increase in the initial concentration of free protons. Proton transport polarization, however, cannot affect β -tricalcium phosphate (β -TCP), which has commonly been used for the preparation of biodegradable composite materials, because it does not contain protons. For this reason, it has been believed up to now that it is impossible to electrically polarize β -TCP ceramics. We have succeeded for the first time, however, in confirming that β -TCP can be electrically polarized. In addition to the adsorption of charge-compensatory ions to the negatively charged surface, large positive surface charges induced by electrical polarization may affect osteogenic cell activity, resulting in enhanced new bone growth [6]. Recent studies indicate that low intensity electrical stimulation is equivalent to various growth factors [7,8]. Moreover, it has been reported that electric and electromagnetic fields enhance regeneration following nerve injury [7–10].

In this study the ability of β -TCP to store an electrical charge induced by electrical polarization was confirmed using thermally

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stimulated depolarization current (TSDC) and electrical conduction measurements. Furthermore, the efficacy of the stored electrical charge on β -TCP particles in enhancing nerve regeneration was evaluated using a nerve bridging model in rats.

2. Materials and methods

2.1. Preparation of β -TCP

β -TCP was manufactured from commercially available $(\text{NH}_4)_2\text{HPO}_4$ and CaCO_3 powders (Wako Pure Chemical Industries, Osaka, Japan). The initial weight ratio of $(\text{NH}_4)_2\text{HPO}_4$ to CaCO_3 was 1.0:1.5. After milling these powders with 70 vol.% ethanol at room temperature for 48 h the ethanol was completely removed using a rotary evaporator. The mixed powders were sintered at 1100 °C for 24 h in air to obtain β -TCP powder, which was mixed into a slurry with 3.0 vol.% polyvinyl alcohol (Wako Pure Chemical Industries) and then spray dried to spherical particles followed by sintering at 500 °C for 30 min to eliminate the polyvinyl alcohol component.

2.2. Characterization of β -TCP

X-ray diffractometry (XRD) was performed on β -TCP particles using a diffractometer control (PW-1710, Phillips, Eindhoven, The Netherlands) fitted with a 4 kW X-ray generator, copper target and graphite monochromator (each $n = 3$). Infrared absorption spectra of β -TCP particles were measured by the KBr pellet method using a Fourier transform infrared (FTIR) spectrophotometer (FTIR-500, JASCO, Tokyo, Japan) in the range 4000–400 cm^{-1} ($n = 3$).

Electrical polarization was carried out according to a previous study [4]. β -TCP particles were put in an alumina ring with the diameter of 2.0 cm and height of 0.5 cm, clamped between a pair of platinum electrodes and electrically polarized in a direct current (DC) field of 4 kV cm^{-1} at 400 °C for 1 h in air (Fig. 1). The samples were cooled to room temperature under electric field polarization. TSDC measurement of the electrically polarized β -TCP particles was performed. The measurements were carried out in air from room temperature to >700 °C at a heating rate of 5.0 $^\circ\text{C min}^{-1}$. The stimulated current was measured with a Hewlett–Packard 4140B pA meter (Palo Alto, CA) and analyzed with the appropriate computer software to calculate the stored electric charge. The Arrhenius equation was plotted from the graph with $\ln \tau(T)$ on

the y-axis and T^{-1} (K^{-1}) on the x-axis, where τ is the orientation time(s) and T is the absolute temperature (K). The gradient was calculated by the method of least squares, giving the energy of activation for relaxation of the electrical polarization.

2.3. Preparation of the chitosan nanofiber mesh tube

The chitosan mesh tube (inner diameter 1.2 mm, thickness – 0.3–0.5 mm) was prepared by electrospinning [2]. Polarized or non-polarized β -TCP particles were immobilized on the tube (15 mg cm^{-2}) by spreading the ethanol suspension. The surface potentials of chitosan nanofiber mesh tubes and those with polarized or non-polarized β -TCP particles were measured using an original Kelvin probe apparatus. Measurements were performed at three evenly separated points along the axis of each tube ($n = 3$ for each tube).

The prepared chitosan nanofiber mesh tubes with polarized or non-polarized β -TCP particles were fixed in 2.5 vol.% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), post-fixed with 1% OsO_4 buffered with 0.1 M PBS and dehydrated through a graded ethanol series. Specimens were dried in a critical point drying apparatus (hcp-2, Hitachi, Tokyo, Japan) with liquid CO_2 , ion sputter coated with platinum and examined by scanning electron microscopy (SEM) (Model S-4500, Hitachi). The size and density of the β -TCP particles immobilized on the chitosan mesh tubes were measured on the SEM images of the composite tubes using Image Pro Plus 6.0 software (Media Cybernetics, Carlsbad, CA) for Windows ($n = 3$ for each group).

2.4. Implantation of the chitosan nanofiber mesh tubes

Male Wistar rats weighing 180–200 g were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1} body weight). The right sciatic nerve was exposed and a 10 mm long section was excised at the center of the thigh. A graft of 15 mm in length was performed by end to end suturing with 8–0 monofilament nylon to connect nerve ends with the following chitosan tubes ($n = 8$ in each group): chitosan mesh tubes with a DAC of 93%; chitosan mesh tubes on which β -TCP particles with or without electrical polarization treatment were immobilized (polarized tube and non-polarized tube, respectively). As a control, portions of sciatic nerve 15 mm in length were harvested from other Wistar rats and grafted into the gap resulting from the resection of a 10 mm segment of the right sciatic nerve in eight rats (isografts).

2.5. Assessment of function recovery

To assess the recovery of motor and sensory function associated with the sciatic nerve, the static toe spread factor (STSF) and von Frey hair test, respectively, were evaluated every 4 weeks until 12 weeks post-implantation.

The test animals were placed on a transparent plastic plate under which a digital camera (Exilim-Z55, Casio, Tokyo, Japan) was set at a distance of 10 cm beneath the surface. After an interval to permit the animal to adapt to the new environment, three frames of both hind feet were taken for each rat. The images were then transferred to a personal computer and the distance between the spread first and fifth toes was determined. Measurements were taken from three consecutive images and averaged for each side. The results were assessed using a simplified formula from the static sciatic index (SSI: scale 1–3):

$$\text{STSF} = \frac{\text{length between the 1st and 5th toes on the normal side} - \text{length between the 1st and 5th toes on the experimental side}}{\text{length between the 1st and 5th toes on the normal side}}$$

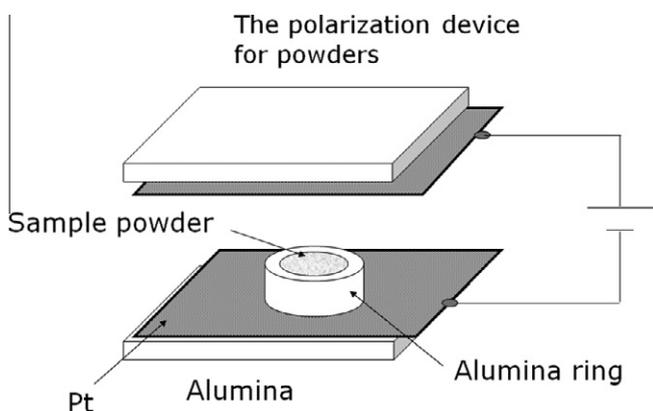


Fig. 1. Scheme of the method to electrically polarize β -TCP particles. The β -TCP particles were put in an alumina ring with a diameter of 2.0 cm and height of 0.5 cm, clamped between a pair of platinum (Pt) electrodes and electrically polarized by applying a DC field of 4 kV cm^{-1} at 400 °C for 1 h in air. The samples were cooled to room temperature under the electric field.

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