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# New nerve regeneration strategy combining laminin-coated chitosan conduits and stem cell therapy

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ABSTRACT

Nerve regeneration remains a difficult challenge due to the lack of safe and efficient matrix support. We designed a laminin (LN)-modified chitosan multi-walled nerve conduit combined with bone marrow stem cell (BMSC) grating to bridge a 10 mm long gap in the sciatic nerve of Sprague–Dawley rats. The repair outcome was monitored during 16 weeks after surgery. Successful grafting of LN onto the chitosan film, confirmed by immunolocalization, significantly improved cell adhesion. In vivo study showed that newly formed nerve cells covered the interior of the conduit to connect the nerve gap successfully in all groups. The rats implanted with the conduit combined with BMSCs showed the best results, in terms of nerve regrowth, muscle mass of gastrocnemius, function recovery and tract tracing. Neuroanatomical horseradish peroxidase tracer analysis of motor neurons in the lumbar spinal cord indicated that the amount and signal intensity were significantly improved. Furthermore, BMSCs suppressed neuronal cell death and promoted regeneration by suppressing the inflammatory and fibrotic response induced by chitosan after long-term implantation. In summary, this study suggests that LN-modified chitosan multi-walled nerve conduit combined with BMSCs is an efficient and safe conduit matrix for nerve regeneration.



## 1. Introduction

Successful rehabilitation in cases of nerve injury is rare because mature neurons do not have the ability to regenerate. However, under the right conditions, self-regeneration can be achieved by axon re-extension over the gap caused by neural injury [1], resulting in reconnection of the proximal and distal stumps and eventually re-establishment of functional connections. Strategies to promote self-regeneration of neurons include supplementation with exogenous growth factors, neutralization of inhibitory factors, replacement of wounded cells and implantation of nerve guidance grafts [2]. Autologous nerve grafting is currently the preferred therapeutic strategy, but the source of the grafts is limited. The use of a donor nerve graft for the transplantation has higher risks of infection and neuroma formation. An alternative material for transplantation is a bioengineered nerve conduit [3,4]. The properties of an ideal nerve conduit include a biodegradable and porous channel wall, the ability to release growth factors, affinity for supporting cells, an internal framework that facilitates cell migration, intraluminal channels that mimic the nerve fascicle structure and electrical conductivity [5].

Among the nerve conduit materials available, chitosan is a natural polysaccharide that is both biocompatible and biodegradable. It is a very versatile material that can be modified to form multifunctional conduits [6–10]. Several techniques have been developed to enhance neural regeneration by mimicking the biological microenvironment [11–13]. For instance, $O_2$ plasma treatment not only renders the material more hydrophilic but also functionalizes the polymer by supplying functional polar groups for covalent conjugation [14]. In terms of biomimetics, biomaterials are combined with extracellular matrix (ECM) proteins to facilitate

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the restoration of the normal structure and function of the damaged tissue. They improve biomaterial integrity and regulate cell activities, including the secretion of the growth factors essential for tissue repair [15]. This is been considered as a suitable option in cell-based therapies aimed at achieving efficient nerve repair [16–18].

Artificial conduit designs have been the subject of ongoing investigations and clinical studies, but no satisfactory solution for nerve repair and functional recovery has emerged yet. Polyesters such as polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) are highly biocompatible, fabricated, have favorable mechanical properties and have the potential to be used in the development of tubular nerve guidance systems. These properties of polyesters have attracted considerable attention [17]. However, the hydrophobicity and poor biological activity of polyesters such as PCL are unfavorable for cell adhesion, and PLGA degradation products can cause a decrease in pH and negative cell growth. In this study, we tested the hypothesis that a new bioartificial conduit containing stem cells could significantly improve the functional recovery of damaged peripheral nerves. We designed a laminin-coated chitosan multi-walled conduit containing bone marrow stem cells (BMSCs) to enhance axonal regeneration. BMSCs are multi-potential cells that are widely used in regeneration medicine because of their stem cell capabilities, such as cell replacement, tropic factor production, ECM protein synthesis and remodeling, microenvironment reconstruction and immune modulation [18,19]. Scaffold, cells and cell regulatory molecules are the three elements essential for regeneration medicine. This study uses in vitro and in vivo approaches to analyze the complex interactions between the elements in this new bioengineered nerve conduit, to evaluate long-term biosafety and efficiency for clinical application.

## 2. Materials and methods

### 2.1. Chitosan membrane preparation

Chitosan (MW 645 KDa; degree of deacetylation $\geqslant$75%) was purchased from Sigma (C3646). About 2 g of chitosan was dissolved in 2% acetic acid, and the solution was treated with hydrogen peroxide for 1 h to eliminate the remaining antigens. After stirring, the solution was filtered through a nylon cloth with medium-sized pores to remove insoluble substances, and the filtrate was lyophilized for preservation. To prepare the chitosan film, the lyophilized chitosan powder was redissolved in 2% acetic acid before being loaded onto a coverslip ($1 \times 1.3$ cm) so that the solvent could evaporate. The films were then soaked in 0.5 M sodium hydroxide solution for 30 min to neutralize the remaining acid. Finally, the films were washed with distilled water and dried.

### 2.2. Hydrophilicity increase and laminin conjugation by electric $O_2$ plasma treatment

The chitosan film was subjected to electric oxygen plasma treatment to increase the hydrophilicity and biocompatibility of the scaffold. The plasma treatment parameters were 36 mTorr $O_2$ pressure and 12 W power for 1 min. After plasma treatment, laminin (100 μg ml$^{-1}$, L-2020; Sigma) was loaded onto the surfaces to bind covalently with chitosan for 2 h, then washed several times with phosphate-buffered saline (PBS).

Laminin (LN) conjugation was demonstrated by luminescent analysis of anti-LN antibody detection. The method followed was the standard Western blot protocol. The films were washed with PBST (0.1 M PBS with 0.1% Tween 20) to rinse off the unbound LN. The LN antibody (1:200; Abcam, ab11575) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:500; Santa Cruz Biotechnology, Inc., sc-2030) were used to mark the remaining bound LN. After washing four times for 10 min in PBST, enhanced chemiluminescence (ECL) was used for antibody detection.

The water contact angles (WCAs) on the original substrate and the treated films were measured by the sessile drop method with distilled water, and observed with a charge-coupled device at room temperature using Microscopi Digitali Dino-Lite (AnMo Electronics Co.). Each drop in the measurement was fixed in 0.9 ml. Each WCA value was the average of four measurements.

### 2.3. Cell culture assay and immunofluorescence

The PC12 cell lines derived from a pheochromocytoma of the rat adrenal medulla were used to investigate the effects of LN-modified materials on adhesion and obtained from ATCC CRL-1722 (Bioresource Collection and Research Center, Hsinchu, Taiwan). For the adhesion test [20], various scaffolds were first immersed in 24-well plates with culturing medium (RPMI-1640 with 10% horse serum, 5% fetal bovine serum (FBS; Biological Industries) and 1% penicillin–streptomycin), and PC12 cells at a cell density of $3 \times 10^4$ cells well$^{-1}$ were seeded into the scaffolds for cultivation for 24 h. The culture medium, along with the suspended cells, was removed before 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was applied to the cell culture for 4 h. The relative number of viable cells was monitored by CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay (MTS) (Promega). For differentiation studies [21], PC12 cells were seeded at $1 \times 10^4$ cells well$^{-1}$ and cultured in low-serum medium (RPMI-1640 medium, 1% horse serum and 1% penicillin–streptomycin), with 50 ng ml$^{-1}$ nerve growth factor (NGF; N-6009, Sigma) being added 24 h later. For immunofluorescence staining [22], cells were fixed using 4% paraformaldehyde at 4 °C, incubated overnight with beta-III-tubulin antibody (diluted to 1:200; Covance) at 4 °C, washed with PBS and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (diluted to 1:400, AlexaFluorR) for 1.5 h at 25 °C. Phalloidin–tetramethyl rhodamine isothiocyanate (Sigma P1951) and Hoechst solution (Sigma H6024) were used to mark the terminal of the neurite and the nucleus, respectively. Stained samples were mounted on glass slides with 50 vol.% glycerol and viewed by fluorescence microscopy (AxioObserver D1, ZEISS).

### 2.4. Bone marrow mesenchymal stem cells

BMSCs were obtained from 4-week-old male Sprague–Dawle rats. Rats were injected with pentobarbital, and BMSCs were collected by flushing nucleated cells out of the femurs and tibiae with PBS. BMSCs were then cultured in T75 flasks coated with poly-D-lysine (Sigma 63103) in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS and 1% penicillin–streptomycin, which were incubated at 37 °C with 5% $CO_2$. Non-adherent cells were removed after 3 days. When 80% confluence was reached, the adherent cells were trypsinized [23]. In this study, only BMSCs within four passages were used for further experiments. The BMSCs were tested with flow cytometry for CD-29, CD-44, CD-90 and CD-34 in order to verify the stability of the stem cells.

### 2.5. Surgical procedure

Adult female Sprague–Dawley rats weighing around 250 g were used in this study. Female rats were chosen instead of the male rats used in most previous studies because the functional evaluation would be less affected by any weight increase. All procedures involving animals were approved by the Animals Committee of the Taipei Veterans General Hospital. The animals were randomly divided into three groups: empty silicone conduit (empty group,