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Research paper

Nerve conduits based on immobilization of nerve growth factor onto modified chitosan by using genipin as a crosslinking agent

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ABSTRACT

Incorporation of nerve growth factor (NGF) into a nerve conduit can improve peripheral nerve regeneration. Here, genipin, a natural and low toxic agent, was used to crosslink chitosan, a natural polysaccharide, and concurrently to immobilize NGF onto modified chitosan, followed by fabrication of chitosan (CS)–genipin (GP)–NGF nerve conduits. MTT test showed that the cell viability of Schwann cells cultured in the conduit extract was not significantly different from that in plain medium. The neurite outgrowth measurement and immunocytochemistry with anti-growth-associated protein-43 and anti-neurofilament indicated that NGF released from CS–GP–NGF nerve conduits retained the bioactivity of stimulating neuronal differentiation of PC12 cells. Fracture strength measurements and vitamin B12 release analysis confirmed that CS–GP–NGF nerve conduits possessed good mechanical properties and adequate permeability. We also investigated the in vitro release kinetics of NGF from CS–GP–NGF nerve conduits by ELISA. The continuous release profile of NGF, within a 60-day time span, consisted of an initial burst that was controlled by a concentration gradient-driven diffusion, followed by a zero-order release that was controlled by a degradation of chitosan matrix. Collectively, CS–GP–NGF nerve conduits had an integrated system for continuous release of NGF, thus holding promise for peripheral nerve repair applications.

1. Introduction

Peripheral nerve repair after traumatic injury remains a common and challenging clinical problem. For severe transection injury, surgical intervention is required to bridge the resulting large nerve gap typically with autologous nerve grafts harvested from another site of the body. To seek alternatives to autologous nerve grafts, a diverse array of natural and synthetic biomaterials has been used to prepare nerve conduits, which can guide regenerating axons from the proximal to distal stump, maintain an optimal environment for nerve regeneration, and protect the regenerating nerve from scar tissue infiltration [1–4]. Chitosan is derived from chitin, a second most abundant polysaccharide in nature, through full or partial deacetylation. As a natural biomaterial with many favorable properties, chitosan has found a wide range of applications in biomedical fields. Recently, chitosan-based nerve conduits are developed for bridging a large gap in peripheral nerves, achieving success to a certain degree [5–8].

To enhance functional outcomes of peripheral nerve regeneration yielded by nerve conduits alone, biochemical cues, such as support cells and growth factors, are usually incorporated into a nerve conduit. Neurotrophic factors are an important class of growth factors that stimulate and control neurogenesis [9,10]. Among neurotrophic factors, nerve growth factor (NGF), as the prototype of the neurotrophin family, has proven to be able to enhance peripheral nerve regeneration [11,12]. Therefore, to prepare NGF-loaded nerve conduits becomes a research concern in the field of peripheral nerve regeneration [13–15].

In this study, we developed a well-defined procedure to prepare chitosan-based, NGF-loaded nerve conduits. NGF was first immobilized onto chitosan in solution by means of crosslinking agents, followed by fabrication of nerve conduits through a technique of injection molding. Although many crosslinking agents, such as glutaraldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether, and toluene-2,4-diisocyanate, have been used for NGF immobilization [16–20], they, especially glutaraldehyde, may impair the biocompatibility of biomaterials due to their cytotoxicity. Compared with traditional crosslinking agents, genipin has less cytotoxicity and better biocompatibility [21,22], because it is derived from geniposide that is one of the ingredients of the fruits of *Gardenia jasmindides* Ellis, a traditional Chinese plant. In this study, genipin was selected as a crosslinking agent to exert two actions: (1) to

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crosslink chitosan for polymer modification and (2) concurrently to immobilize NGF onto modified chitosan [23].

After chitosan-based, NGF-loaded nerve conduits were prepared, we tested their cytotoxicity and biocompatibility in vitro, evaluated their permeability and mechanical properties, and determined the release kinetics of NGF from them. These experimental data will aid in vivo applications of these newly developed nerve conduits in peripheral nerve repair.

2. Materials and methods

2.1. Fabrication of new nerve conduits

Chitosan powder, with a degree of deacetylation of 92.3% and an average molecular weight of 2.8×10^4, was received from Nantong Xincheng Biochemical Company, Jiangsu, China. The 0.3 g of chitosan powder was dissolved in 20 ml of 10 g/l acetic acid aqueous solution and stirred to form a white viscous solution 15 g/l concentration. The chitosan solution was allowed to react with 4 mM genipin (Seebio Co., Shanghai, China) at 37 °C to generate a genipin-crosslinked chitosan (CS–GP) solution. Otherwise, 4 mM genipin and 0.4 μg/ml NGF were sequentially added to the chitosan in acetic acid solution and allowed to react at 37 °C to generate a chitosan–genipin–NGF (CS–GP–NGF) solution.

To prepare nerve conduits by an injection molding technique, we used a stainless-steel casting mold consisting of an inner pillar (φ = 3 mm) and an outer tube (φ = 5 mm), both of which were fixed on the mold bottom and determined the diameter and thickness of conduits. The above-mentioned CS–GP or CS–GP–NGF solution was injected into the mold immediately following preparation. The solution in the mold underwent a transformation into a gel-like state due to genipin crosslinking and further solidification for 24 h, followed by demolding under lyophilization (−56 °C, 6 h) to furnish CS–GP or CS–GP–NGF nerve conduits, respectively.

The CS–GP membrane was prepared by spreading the CS–GP solution onto a glass plate under sterilization conditions and allowing the solution to be transformed into a gel-like state and further dried at room temperature. The resulting membrane was peeled, washed, and cut into round pieces (φ = ∼12 mm) for use. For preparing the CS membrane, the chitosan in acetic acid solution (without genipin added) was dried to form the membrane, followed by addition of 4% sodium hydroxide for 4 h to neutralize the acid.

All prepared products were disinfected with 70% alcohol and washed with phosphate buffered saline (PBS, 0.1 M, pH 7.4) prior to use.

2.2. Scanning electron microscopy

Morphology of CS–GP–NGF conduits was examined under a Philips XL-30 Scanning Electron Microscope (SEM, Eindhoven, Netherlands). The sample was coated with gold using a JEOL JFC-110E Ion Sputter before examination under SEM.

2.3. Measurements of crosslinking degree and swelling ratio

The crosslinking degree of the CS–GP membrane was determined by the ninhydrin assay as described previously [24]. In brief, the lyophilized sample was placed in a ninhydrin solution (2% w/v) for 20-min heating at 100 °C, and the absorbance was recorded at 570 nm by spectrophotometry. The CS membrane served as a control. We calculated the crosslinking degree of samples by the equation: crosslinking degree (%) = $[(\text{NH reactive amine})_{\text{fresh}} - (\text{NH reactive amine})_{\text{fixed}}]/(\text{NH reactive amine})_{\text{fresh}} \times 100$, where fresh and fixed were the mole fraction of free NH_2 remaining in non-crosslinked and crosslinked samples, respectively.

The dried CS–GP membrane was weighed to give W_1 (initial weight). Then the membrane was immersed into PBS for 24-h swelling at room temperature. After the swollen membrane was blotted with a piece of tissue to absorb excess water on the surface, it was weighed to give W_2, (swollen weight). Then it was air dried several times until reaching a constant weight, which was designated as W_3. The swelling ratio and sol fraction of the CS–GP membrane were respectively calculated by two equations:

$$\text{Swelling ratio } (\%) = [(W_2 - W_3)/W_3] \times 100$$

$$\text{Sol fraction}(\%) = [(W_1 - W_3/W_3)] \times 100$$

2.4. Schwann cell culture

After sterilization, the CS–GP–NGF or CS–GP nerve conduit was cut into small pieces (about 1 mm long), which were placed in an extraction container. Then, L15 basic medium (Gibco, Grand Island, NY) was added, according to the proportion of 1 g conduit piece to 10 ml medium, for 72-h incubation at 37 °C to generate two extracts. In addition, the extract of organotin powder (from Hongding Chemicals Company, Nantong, Jiangsu, China) in L15 medium was prepared in the similar manner, serving as a negative control.

To harvest Schwann cells, the bilateral sciatic nerves of neonatal Sprague–Dawley rats (1–2 days old) were excised out under sterile condition. After the epineurium and perineurium were removed from sciatic nerves, the sciatic nerves were sheared into 0.01 mm^3 tissue fragments, which were then planted onto a polylysine-coated coverslip in a 6-well plate. The medium of L15 supplemented with 15% fetal bovine serum (FBS) was added to the plate (2 ml/well), followed by incubation at 37 °C in a humid 5% CO_2 atmosphere. Half of the medium was replaced every other day.

After 7-day incubation, primary cultured Schwann cells were moved to a 96-well plate at a cell density of 1×10^4/well. The above prepared three extracts and plain L15 medium (serving as positive control) were added to the plate, respectively, to allow for culture of Schwann cells. At different time intervals, the cell viability of Schwann cells was examined by MTT assay as previously described [25].

2.5. PC12 cell culture

Rat PC12 cells, obtained from the American Type Culture Collection (Manassas, VA), were seeded on a 24-well culture dish at the density of 3×10^4 cells/well for incubation in nutrient mixture F12 Ham Kaighn's modification (F12K) medium (Gibco) supplemented with 15% heat-inactivated horse serum, 2.5% FBS and antibiotics (Gibco) in a humid 5% CO_2 atmosphere.

2.5.1. Lactate dehydrogenase (LDH) leakage assay

Genipin at different concentrations (2, 4, and 8 mM) was added to the chitosan solution, respectively, for 24-h reaction at 37 °C, thus generating CS–GP membranes with different crosslinking degrees, which were abbreviated as CS–GP2, CS–GP4, and CS–GP8 membranes. PC12 cells at the density of 3×10^4 cells/well were seeded on these membranes in 24-well culture dishes to allow incubation in plain F12K culture medium for different times (1, 3, and 7 days). Then PC12 cells were subjected to LDH test with a LDH cytotoxicity assay kit (Cayman Chem. Co., Ann Arbor, MI) according to the kit instructions. Here, LDH test was adopted to evaluate the cytotoxicity of CS–GP membranes to PC12 cells. As is known, LDH is a soluble cytosolic enzyme that is delivered into the culture medium following a loss of membrane integrity

resulting from either apoptosis or necrosis of cells, so LDH leakage is an indicator of cell damage [26].

2.5.2. Neurite outgrowth of PC12 cells

Since PC12 cells differentiate toward a neuronal phenotype in response to stimulation by NGF, the bioactivity of NGF releasing from the CS–GP–NGF nerve conduit can be evaluated according to the neurite outgrowth from PC12 cells. The CS–GP–NGF or CS–GP nerve conduit was placed in an apical chamber of a transwell system (Corning Incorporated, Corning, NY), respectively, while PC12 cells were seeded on the basal chamber at a density of 3×10^4 cells/well and cultured in plain F12K culture medium. The above two experimental settings were abbreviated as CS–GP–NGF and CS–GP groups, respectively. In addition, PC12 cells in the basal chamber of the transwell system were cultured in plain F12K culture medium added with or without 50 ng/ml NGF, while the apical chamber was unoccupied, which were abbreviated as +NGF or −NGF groups serving as positive and negative controls, respectively.

Following the above cell culture for different times, the percentage of neurite-bearing cells was determined by counting 200 cells in randomly selected fields with a digital camera (Leica, Cambridge, England) and a computerized image analyzer (Leica). Neurite-bearing cells are those with processes greater than or equal to the diameter of the cell body.

2.5.3. Immunocytochemistry

In an alternative set of tests, following the above cell culture for different times, PC12 cells in the basal chamber were taken out, and fixed with 4% paraformaldehyde in PBS, followed by incubation in a solution containing 10% goat serum and 3% BSA at room temperature to block nonspecific binding. Then, they were allowed to incubate with primary antibodies, i.e., goat anti-growth associated protein-43 (GAP-43, 1:200 dilution, Santa Cruz, CA) and rabbit anti-neurofilament (NF) 200 IgG fraction of antiserum (1:200 dilution, Sigma), at 4 °C in humid 5% CO_2 atmosphere overnight. After wash with PBS, the slides were further reacted with second antibodies, i.e., FITC-labeled donkey anti-goat IgG (1:200 dilution, Santa Cruz) and Cy3-labeled goat anti-rabbit IgG (1:200 dilution, Santa Cruz), for 2 h at 37 °C. Then, the samples were mounted in fluorescent mounting medium and observed under a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany).

2.6. Mechanical property measurement

The tensile strength or transverse compression strength of CS–GP–NGF nerve conduits was measured on a J-100N strength-testing machine after they were or were not soaked in PBS at 37°C. The nerve conduit was cut into 50 mm in length. The wall thickness of nerve conduits was pre-measured. The tensile speed and gauge length were set as 10 mm/min and 40 mm, respectively. The crosshead speed was maintained at 1.0 mm/min.

2.7. Permeability test

The CS–GP–NGF nerve conduit, cut into a 10-mm-long specimen, was filled with 0.02 ml of vitamin B12 (Sigma) aqueous solution with the concentration of 0.2 mg/ml, sealed with candlegrease, and stored in a 20 ml of distilled water at 37 °C [27]. The release medium, i.e., distilled water, was replaced at different time intervals. The concentration of vitamin B12 was determined by UV spectroscopy at 550 nm using a U-3000 spectrophotometer (Hitachi, Osaka, Japan). The cumulative release percentage of vitamin B12 was calculated according to the formula: the cumulative release percentage = the concentration of B12 in distilled water at one time point/the theoretic concentration of vitamin B12.

2.8. NGF release kinetics

The amount of NGF released from the CS–GP–NGF nerve conduit was analyzed by ELISA. The CS–GP–NGF nerve conduit was incubated in PBS at 37 °C. At each time interval, the PBS was collected and then replaced with fresh PBS. The collected PBS was stored at −20 °C for determining the released amount of NGF with a sandwich ELISA kit (Chemicon, Temecula, CA) according the kit instructions. The NGF antibody in the kit specifically reacted with the beta-subunit of NGF in the 7S form and the total NGF was thus detected.

2.9. Statistical analysis

All analyses were performed using a standard Student's *t*-test. Statistical significance was considered for $p < 0.05$. Data were expressed as means ± SD. Unless otherwise indicated, measurements were performed in triplicate.

3. Results and discussion

Chitosan is one of excellent natural biomaterials for preparing porous nerve conduits with many advantages including biocompatibility, chemical versatility, and controlled degradability. Genipin, an agent of natural origin, has been used to fix biological tissues and to crosslink amino-group-containing polymers. Genipin is superior to traditional crosslinking agents in terms of lower cytotoxicity. Also it has proven to have neuritogenic activity in PC12h cells (a subclone of PC12 cells) and neuroprotective effects on b-amyloid peptide-treated primary hippocampal neurons of rats that express nNOS proteins [28]. As a result, genipin has been used to crosslink growth factors with polymers in tissue engineering fields. To our knowledge, however, few reports are involved in crosslinking of NGF with chitosan-based nerve conduits. In this study, we developed novel CS–GP–NGF nerve conduits and evaluated their characteristics. In our design, the crosslinking reaction took place in the process of genipin-induced chitosan modification and also in the process of NGF immobilization onto modified chitosan.

3.1. The morphological observation of CS–GP–NGF nerve conduits

The morphology of CS–GP–NGF nerve conduits was observed by SEM. The dense outer layer of conduits could provide mechanical support and strength to CS–GP–NGF nerve conduits, and also exerted a barrier function by preventing ingrowth of fibrous tissues, while the sponge-like inner layer of conduits could allow the exchange of nutrition and fluids, and afford a massive space for the storage of the released NGF (Fig. 1).

3.2. Optimization of genipin concentration for crosslinking

The crosslinking degrees, swelling ratios, and sol fractions of CS–GP membranes at different genipin concentrations were measured, and the data are listed in Table 1. The crosslinking degree increased with the genipin concentration increasing, but the swelling ratio or sol fraction decreased as the genipin concentration increased.

LDH leakage data at different culture times showed that the cytotoxicity to PC12 cells had no significant difference at 2 and 4 mM of genipin concentration, but showed an increasing trend at 8 mM of genipin concentration (Fig. 2A). The reason for this

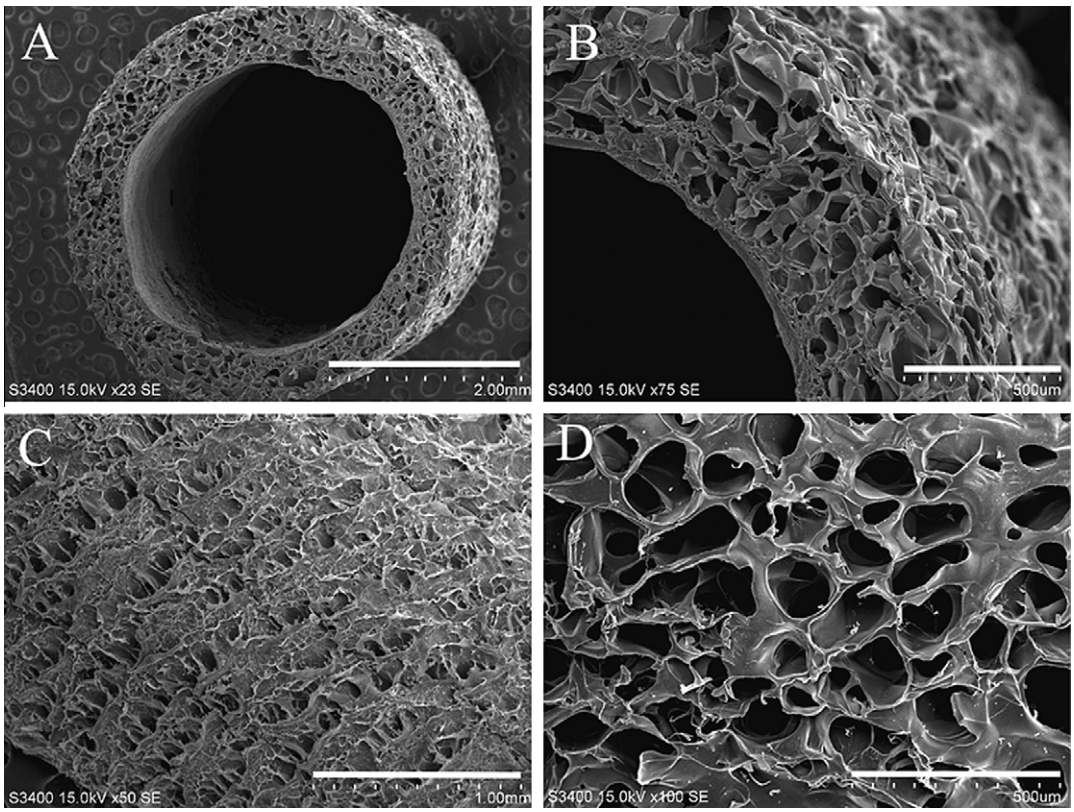

Fig. 1. SEM images of CS–GP–NGF nerve conduits. Side view of the conduit (A), cross-section view of conduit (B), inner layer of conduit wall (C), and outer layer of conduit wall (D). Scale bar, 2000 (A), 1000 (C), and 500 μm (B and D).

Table 1
The effects of the genipin concentration on the crosslinking degree, swelling ratio and sol fraction of CS–GP membranes, in which the data were expressed as means ± SD (n = 6).

Genipin concentration (mM)	Crosslinking degree (%)	Swelling ratio (%)	Sol fraction (%)
0.5	N/A	178.8 ± 6.3	60.5 ± 8.5
1.0	33.0 ± 3.2	37.7 ± 4.0	29.5 ± 5.8
2.0	45.9 ± 3.3	16.1 ± 4.7	22.3 ± 4.4
3.0	50.5 ± 2.9	14.5 ± 2.4	16.7 ± 4.2
4.0	61.9 ± 1.9	13.4 ± 5.7	8.3 ± 1.8
5.0	70.4 ± 1.7	8.8 ± 2.1	4.7 ± 0.9
8.0	75.6 ± 1.2	8.4 ± 1.5	4.2 ± 0.8

might be that increase in the rigidness of the membrane CS–GP8, induced the higher genipin concentration, caused the formation of broken pieces, and thus increased the cell toxicity. Taking into account of both swelling analysis and LDH test, we selected 4.0 mM genipin for preparing CS–GP or CS–GP–NGF membranes or nerve conduits.

3.3. In vitro biocompatibility evaluation of CS–GP–NGF nerve conduits

3.3.1. In vitro cytotoxicity to Schwann cells

After cell culture for 12, 48, 72, or 168 h, the viability of Schwann cells in the CS–GP–NGF or CS–GP extract was not significantly different from that in plain L15 medium (positive control), while the viability of Schwann cells cultured in the organotin extract (negative control) was significantly smaller than that in the above three mediums (Fig. 2B). The comparisons indicated that CS–GP–NGF nerve conduits were low-cytotoxic to Schwann cells, an important type of glial cells in the peripheral nerve system.

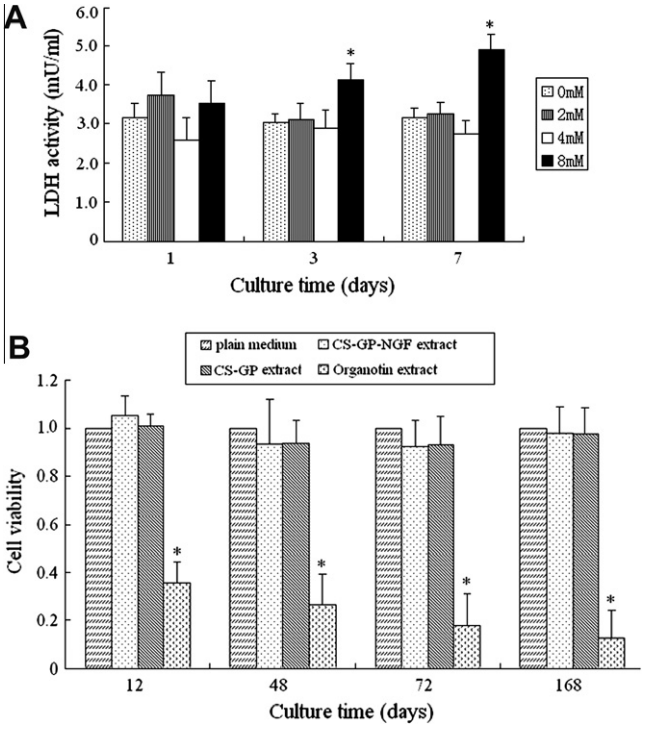

Fig. 2. (A) LDH activity of PC12 cells culture on CS–GP, CS–GP2, CS–GP4, and CS–GP8 membranes for different times (1, 3, and 7 days). *$p < 0.05$ vs. other membranes. (B) The changes in the cell viability of Schwann cells, measured by MTT assay, after they were cultured in plain L15 medium (positive control), CS–GP nerve conduit extract, CS–GP–NGF nerve conduit extract, and organotin extract (negative control) for 12, 48, 48, and 168 h, respectively. The cell viability was expressed as a value relative to positive control. *$p < 0.05$ vs. other three culture mediums.

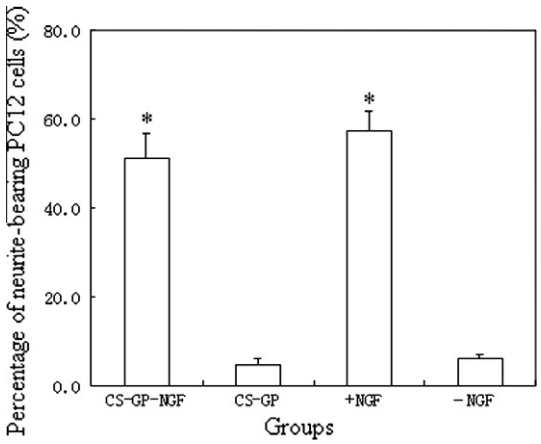

Fig. 3. The percentage of neurite-bearing PC12 cells in whole population after 7 day culture in four different groups. $^{*}p < 0.05$ vs. CS–GP group or −NGF group.

3.3.2. NGF-stimulated differentiation of PC12 cells

The neurite outgrowth of PC12 cells was investigated by counting the number of neurite-bearing PC12 cells (differentiated PC12 cells). After 7 day culture, the neurite growth in CS–GP–NGF group was a little less than that in +NGF groups without significant difference, but the neurite growth in CS–GP–NGF group was significantly greater than that in either CS–GP or −NGF group (Fig. 3). The result suggested that the released NGF from CS–GP–NGF nerve conduits kept its bioactivity, i.e., its ability to promote neurite outgrowth from PC12 cells.

After culture for different times, PC12 cells in four different groups underwent immunocytochemistry with antibodies against GAP-43 and NF200 (separately and merge). Since GAP-43 is an axonal membrane protein involved in the neuronal outgrowth and synaptic plasticity of developing neurons [29], and NF200 is commonly used as a neuronal cell marker, the immunocytochemistry indicated that NGF-stimulated differentiation of PC12 cells toward the neuronal cell phenotype in CS–GP–NGF group was

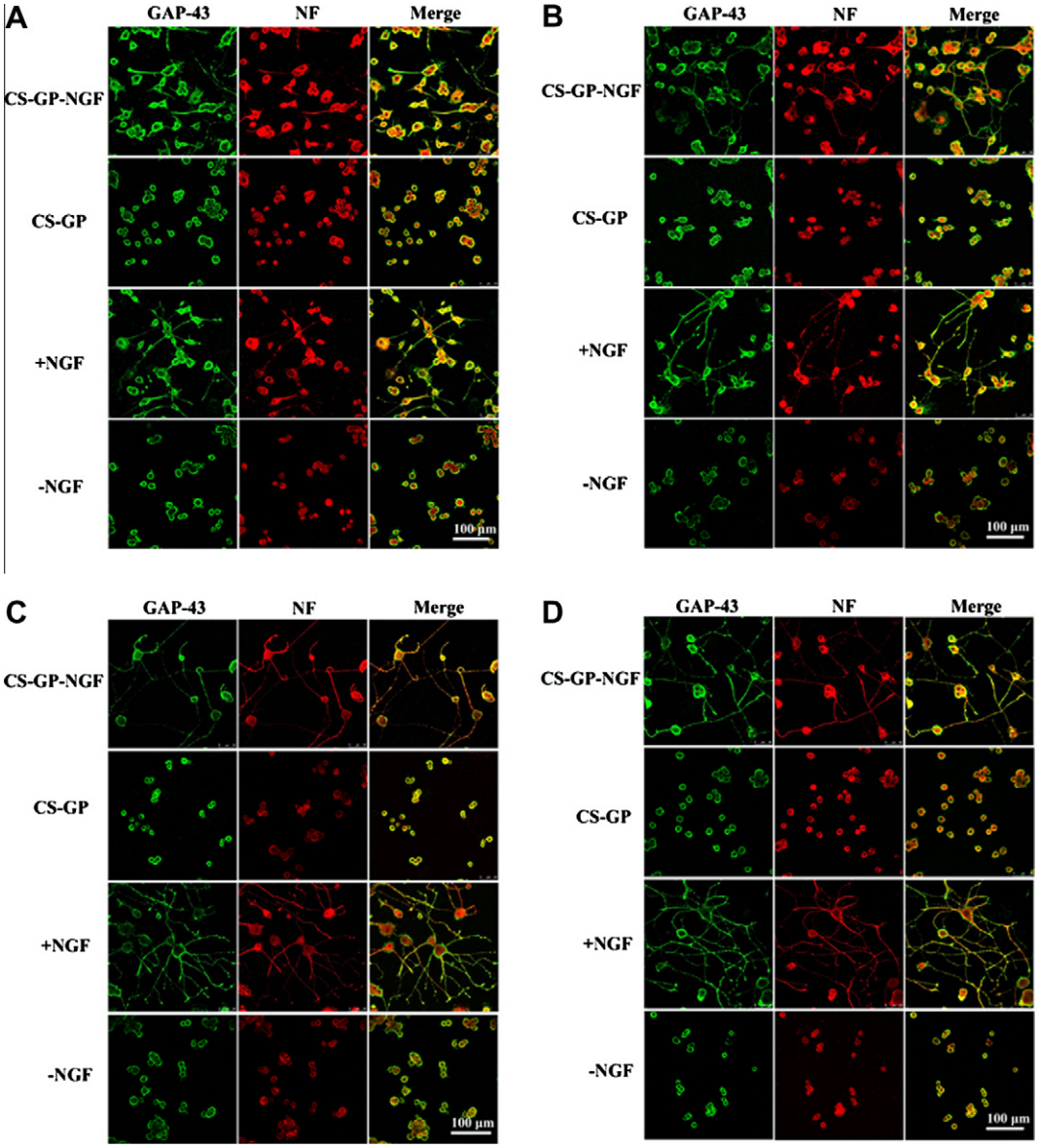

Fig. 4. Immunocytochemistry with anti-GAP-43 and anti-NF200 of PC12 cells in four different groups after they were cultured for 3 (A), 5 (B), 7 (C), and 10 (D) days. Scale bar, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

close to, albeit a little less than, that in +NGF group, and this neuronal differentiation in both CS–GP–NGF and +NGF groups was obviously better than that in CS–GP or –NGF group at all designated time intervals (Fig. 4).

Taken together, the results collectively suggested that the released NGF from CS–GP–NGF nerve conduits retained its basic biological activity, which was similar to that of free NGF.

3.4. The mechanical property and permeability of CS–GP–NGF nerve conduits

The maximum fracture strength of CS–GP and CS–GP–NGF nerve conduits was measured to be 5.9 ± 0.3 and 8.7 ± 0.5 N, respectively, under wet conditions. These data indicated the cross-linking of NGF to chitosan by genipin did not reduce but, on the contrary, enhance the maximum fracture strength of chitosan material.

We also measured the permeability of CS–GP–NGF and CS–GP nerve conduits by examining the release profile of vitamin B12 (molecular weight 1355), a model nutrient, which had been loaded into sealed conduits and allowed to release into water outside. The data indicated that the release rate increased with time for both types of nerve conduits, and both types of nerve conduits showed no significant difference in the permeability (Fig. 5). The permeability of CS–GP–NGF nerve conduits could meet the requirements for material exchange during peripheral nerve regeneration.

3.5. NGF release profile from CS–GP–NGF nerve conduits

The amount of NGF released from CS–GP–NGF nerve conduits was analyzed by ELISA until 60 days. The burst release happened during the initial stage and slowed down thereafter. NGF was constantly released from CS–GP–NGF nerve conduits and could be traced until 60 days. The release curve showed two distinctive parts with different slopes, which might represent two different mechanisms (Fig. 6): at the first 3 days, 2.1 ng/day of NGF, in average, was released from CS–GP–NGF nerve conduits; from day 4 to day 25, 0.4 ng/day of NGF, in average, was released from the conduit; at day 60, the minimum daily release of NGF was 0.22 ng/day in average. Based on this release profile, the NGF amount released from CS–GP–NGF nerve conduits seemed to be suitable to promoting peripheral nerve regeneration.

It seemed that at the initial stage, NGF released from CS–GP–NGF nerve conduits might be controlled by concentration gradient-driven diffusion; later the NGF release followed zero-order kinetics in terms of the nearly constant release rate, and the zero-order kinetics was controlled by degradation of chitosan. During the degradation of chitosan, genipin molecules on the chitosan surface might strongly trap the amino groups of NGF, causing a decrease in the release rate.

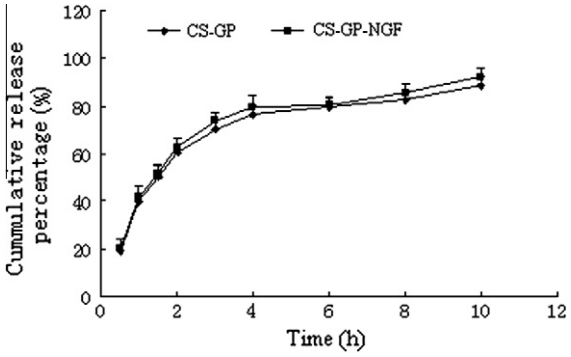

Fig. 5. Cumulative release of vitamin B12 from CS–GP–NGF and CS–GP nerve conduits.

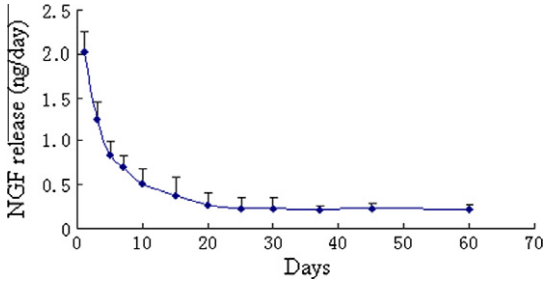

Fig. 6. Daily in vitro release of NGF from the CS–GP–NGF nerve conduit to PBS (pH 7.4) at 37 °C. The conduit was 20 mm long, prepared with about 300 μl CS–GP–NGF solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

We prepared NGF-loaded, chitosan-based nerve conduits in which NGF molecules were successfully immobilized on genipin-modified chitosan. These CS–GP–NGF nerve conduits were non-cytotoxic to primary cultured Schwann cells and showed in vitro neuro-affinity to PC12 cells in terms of keeping the activity of NGF within nerve conduits, i.e., the ability to stimulate neuronal differentiation of PC12 cells. The mechanical properties and permeability of CS–GP–NGF nerve conduits were examined by fracture strength measurements and vitamin B12 release analysis. More intriguingly, the continuous release profile of NGF from CS–GP–NGF nerve conduits, within a 60-day time span, consisted of an initial burst that was controlled by concentration gradient-driven diffusion, followed by a zero-order release that was controlled by the degradation of chitosan.

To sum up, in vitro characterization and evaluation of CS–GP–NGF nerve conduits inspire us to improve the design of CS–GP–NGF nerve conduits and develop their applications in peripheral nerve regeneration.

Disclosure

There is no conflict of financial interest involved in this study.

Acknowledgments

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