

Biocompatibility of NGF-grafted GTG membranes for peripheral nerve repair using cultured Schwann cells

Pei-Ru Chen^a, Ming-Hong Chen^{a,b}, Jui-Sheng Sun^{c,f}, Mei-Hsiu Chen^d, Chien-Chen Tsai^e,
Feng-Huei Lin^{f,*}

^a*Institute of Biomedical Engineering, College of Medicine, College of Engineering, National Taiwan University, No 1, Sec. 1, Jen-Ai Road, Taipei, Taiwan*

^b*Division of Neurosurgery, Department of Surgery, Cathay General Hospital, Taiwan*

^c*Department of Orthopedics, Yunlin Hospital, Taiwan*

^d*Department of Medicine, Far Eastern Memorial Hospital, Taiwan*

^e*Department of Pathology, Far Eastern Memorial Hospital, Taiwan*

^f*Department of Biomedical Engineering, National Taiwan University Hospital, Taiwan*

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Abstract

We previously developed a biodegradable composite with potentially good biocompatibility composed by tricalcium phosphate and glutaraldehyde cross-linking gelatin (GTG) with good mechanical property feasible for surgical manipulation. The purpose of this study was to evaluate the feasibility of immobilizing nerve growth factor (NGF) onto the composite (GTG) with carbodiimide (GEN composite). Cultured Schwann cells were seeded onto the GTG and GEN composites. For comparison, GTG membrane soaked in NGF solution without carbodiimide (GN composite) as cross-linking agent was also used to culture Schwann cells. Cell morphology was observed by a scanning electron microscope. Cell survival, cytotoxicity and cellular metabolism on the NGF-grafted GTG membrane were assessed quantitatively in terms of cell protein content, leakage of cytosolic lactate dehydrogenase (LDH) activity and by the well-established MTT assay, respectively. The result of LDH study did not show significant difference among GTG, NGF-modified GTG and control group. This indicated that GTG composite, whether cross-linking with NGF or not, has little cytotoxic effect. Comparing the protein content and MTT assay among GEN, GN composite and control group, the data confirmed more attachment of Schwann cells on GEN composite. Although GTG cross-linking with NGF did not promote Schwann cell proliferation, the techniques we used in this study provided a method to fabricate a novel biomaterial incorporation of Schwann cells and covalently immobilized NGF.

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Keywords: Biodegradable nerve conduit; Gelatin; Schwann cell; Nerve growth factor

1. Introduction

In clinical practice, direct end-to-end suturing techniques are suggested for a short nerve injury. When large gaps remain between the ends of injured peripheral nerves, nerve autografting has been the first choice for repairing nerve gaps. However, this approach has inevitable disadvantages, such as limited supply of available nerve autografts and certain donor site

morbidity. Xenografts and allografts have been evaluated as alternatives to autografts but have poor successful rate and problems of immune rejection [1,2]. A wide range of materials has been developed for use as a synthetic nerve guidance channel [3–8]. Of these materials, nondegradable materials such as silicone rubber have been widely used because of their inert and mechanical properties. However, upon completion of regeneration these materials no longer serve any purpose and may become detrimental due to mechanical impingement or infection [9]. Contrarily, biodegradable materials potentially avoid these problems and make secondary surgery unnecessary. Biodegradable materials such as polyglycolic acid (PGA) [1] and polylactic acid

*Corresponding author. Tel.: +886-2-23912641; fax: +886-2-23940049.

E-mail address: double@ha.mc.ntu.edu.tw (F.-H. Lin).

(PLA) [10,11] are used for tubulization to bridge both ends of the amputated peripheral nerve. Nonetheless, upon degradation by hydrolysis, PLA and PGA produce lactic acid and glycolic acid, respectively. These degradation by-products would lead to a lowering of local pH and could be detrimental to the surrounding cells and tissue [12,13]. Nerve guidance channels have also been fabricated out of collagen with favorable results in nerve repair [5,14,15]. But collagen is rather expensive and difficult to handle during suturing because of mechanical weakness. On the other hand, thermal denaturation or physical and chemical degradation of collagen gives gelatin. With respect to collagen, gelatin does not express antigenicity in physiological conditions, and it is much cheaper and easier to obtain in concentrate solutions. Besides, gelatin is a biodegradable polymer with excellent biocompatibility, plasticity and adhesiveness [16]. The use of proper cross-linking agents such as glutaraldehyde at low concentration further allows to modulate the mechanico-chemical properties of gelatin films [17].

When designing a nerve guidance channel from tissue engineering approach, besides conduit material, incorporation of neurotrophic factors and cells supporting axon regeneration should also be considered. Nerve regeneration has been shown to be enhanced across gaps utilizing guidance channels filled with neurotrophic factors such as nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF) and neurotrophin NT-3 [18–21]. On the other hand, gelatin contains primary carboxyl groups on its chain backbones which give gelatin potential to bind covalently the amine groups of these neurotrophic molecules with appropriate cross-linking agent [22–25]. We previously developed a biodegradable and biocompatible composite composed by tricalcium phosphate and glutaraldehyde cross-linking gelatin (GTG) with good mechanical property feasible for surgical manipulation. In this work, nerve-growth-factor (NGF) was immobilized onto the surface of GTG membrane (GEN). Cross-linking with a water-soluble carbodiimide led to a covalent and stable binding between the amine groups of NGF and the carboxyl groups on the surface of GTG membrane.

Besides NGFs, several studies have shown enhanced regeneration when Schwann cells were seeded within nerve guidance channels [26–30]. Therefore, we also tried to culture Schwann cells on our composite membrane. Then, biocompatibility of the composite was assessed quantitatively in terms of cell protein content, leakage of cytosolic lactate dehydrogenase (LDH) activity [31] through damaged Schwann cell membranes and by the well-established MTT assay [32]. In this study, the morphology of Schwann cells seeded on the GEN membrane and the results of *in vitro* biocompatibility studies suggest that our tissue engi-

neered composite membrane is a promising candidate biomaterial for nerve regeneration.

2. Materials and methods

2.1. GTG membrane preparation

We prepared the matrix phase of the GTG composite by adding 22.5 g of bovine gelatin (Sigma Chemical Co., St. Louis, MO, USA) to 380 ml of deionized distilled water. The mixture was stirred vigorously and kept at 70°C until a homogenous gelatin solution was attained. 22.5 g of TCP particle were then poured down into the gelatin solution. The mixture was stirred for 5 min to ensure a uniform consistency. In order to obtain the homogenous and higher cross-linking density of the composites, the temperature of the mixture was cooled to 35°C, then, 22.5 ml 1% glutaraldehyde solution was added for cross-linking reaction of the gelatin. After the mixtures were cross-linked, they were poured down into blank dishes and dried in an oven overnight to form membranes. The membranes were then soaked in 0.1% glutaraldehyde solution for another 8 h for further cross-linking. The cross-linked GTG membrane was further treated with 0.1 M glycine aqueous solution to block non-reacted aldehyde groups, and then washed with double-distilled water. The composites were used in the later experiment for Schwann cell seeding and NGF grafting.

2.2. NGF covalently immobilized onto GTG membrane (GEN membrane)

To immobilize NGF (Chemicon Co., 7S NGF) onto GTG membrane, GTG membranes were soaked in 20 ml 0.1 wt% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) solution for 24 h at 4°C. The membranes were then transferred to 50 ng/ml NGF solution for 24 h at 4°C. The reaction of NGF with the primary amine that existed on the surface of GTG membranes was initiated with carbodiimide. The proposed mechanism of NGF cross-linking is shown in Fig. 1. Unbound and excess chemicals were removed by washing the membranes with phosphate buffer solution (PBS).

2.3. Cell culture

GTG/GEN membranes were cut into circular discs suitably sized for tissue culture plate wells. The membranes were sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively with PBS. Membranes for this experiment were divided into four groups for Schwann cell culture ($n = 18$): (1) GTG membrane with NGF covalently immobilized

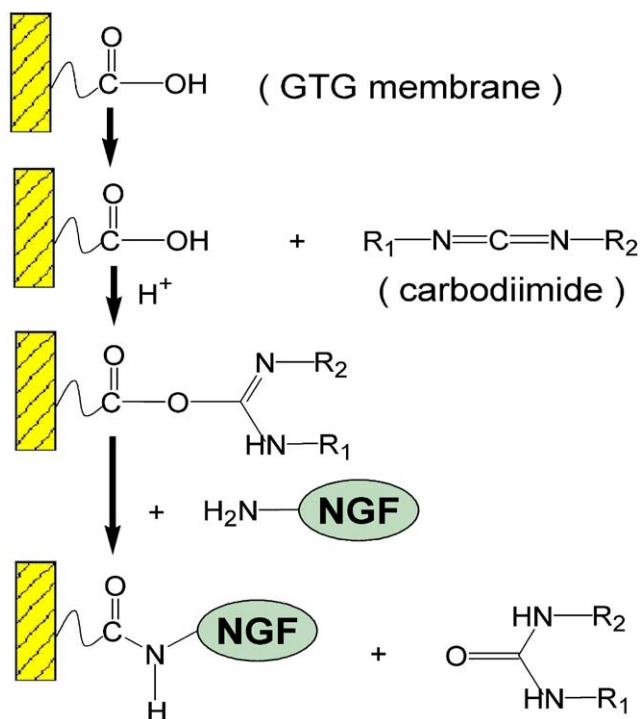


Fig. 1. The steps of cross-linking with carbodiimide led to a covalent and stable binding between the amine groups of NGF and the carboxyl groups on the surface of GTG membrane.

(GEN), (2) GTG membrane soaked in 50 ng/dl NGF solution for 24 h (GN) (washed three times with PBS before cell culture), (3) blank GTG composite (GTG), and (4) Petri dish as control group.

Schwann cells from neonatal Lewis rats were isolated and expanded in culture using methods described by Brockes et al. [33]. Briefly, 4 litters of day 2 Lewis pups were killed, and their sciatic nerve were removed. After enzymatic digestion with collagenase and dispase, the primary cell suspension was plated in Dulbecco modified Eagle medium (DMEM), enriched with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic (penicillin–streptomycin solution). The cells were treated for the ensuing 2–3 days with cytosine arabinoside to inhibit the proliferation of fibroblast. On the 7th day, 0.2 ml 5×10^5 cells/ml Schwann cells were seeded in individual wells of a 24-well tissue culture plate with 2 ml DMEM and 10% FBS for testing the effects of various GTG composites on the growth of Schwann cells. The cultures were examined by using light microscope on a daily basis for observations of adherence, survival and proliferation.

2.4. Morphological evaluation of Schwann cells cultured on GTG composites

For morphological evaluation, the Schwann cells adhering to the GTG membrane were fixed with 10%

formaldehyde in PBS for 24 h. After washed with PBS, the cell seeded membrane were dehydrated through an ascending alcohol series, commencing at a solution of 70% alcohol, then dried in a critical point drier (CPD) and gold coated. All specimens were then examined by scanning electron microscope.

2.5. Evaluation of biocompatibility of GTG membrane

On day 1, 3, 7 after Schwann cells seeded onto composite membranes in each experimental group, biocompatibility of the composite was assessed quantitatively in terms of cell protein content, leakage of cytosolic LDH activity and by the MTT assay.

2.5.1. Total cell protein

Eighty microliters of 0.5 M NaOH were added to each well, the plates were sealed and cell proteins digested at 37°C for 18 h. 0.2 ml diluted medium was mixed with 2.2 ml diluted biuret reagent and later with 0.1 ml Folin and Ciocalteu's Phenol Reagent (Sigma) [34]. The color formed was read at a wavelength of 700 nm. Protein concentrations were determined from a calibration curve.

2.5.2. LDH leakage

LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) resulting in an increase in absorbance at 340 nm. Schwann cell injury was quantitatively assessed by the measurement of LDH released from damaged or destroyed cells, in the extracellular fluid. Ten microliters of aliquots of the medium was mixed with 200 μ l LDH reagent (Sigma). LDH activity was calculated by measuring the increase in absorbance at 340 nm, as described previously [35].

2.5.3. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was prepared as 0.5 mg/ml stock solution in PBS [36]. MTT (0.35 ml) was added to each well and plates were incubated for 4 h at 37°C. After incubation, the medium was aspirated and the formazan reaction products were dissolved in 100 μ l 0.04 N HCl/EtOH. The optical density of the formazan solution was measured on an ELISA plate reader (Labsystems Multiscan RC) at 570 nm.

2.6. Statistical analysis

In this study, all experiments were performed in a paired pattern and replicated 10 times. The differences between various tested conditions were evaluated by the paired *t*-test. The level of statistical significance is defined as $p < 0.05$.

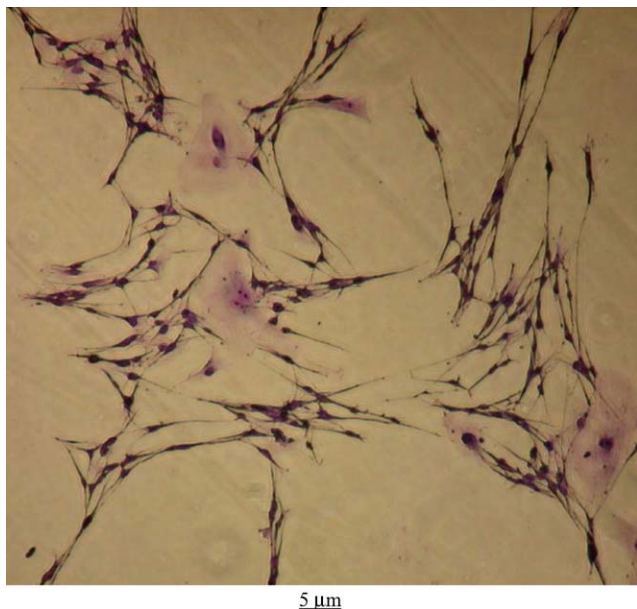


Fig. 2. The optical microscopic examination of the Schwann cell cultured in the control group (100 \times). The Schwann cell adhered and extended processes well on the Petri dish.

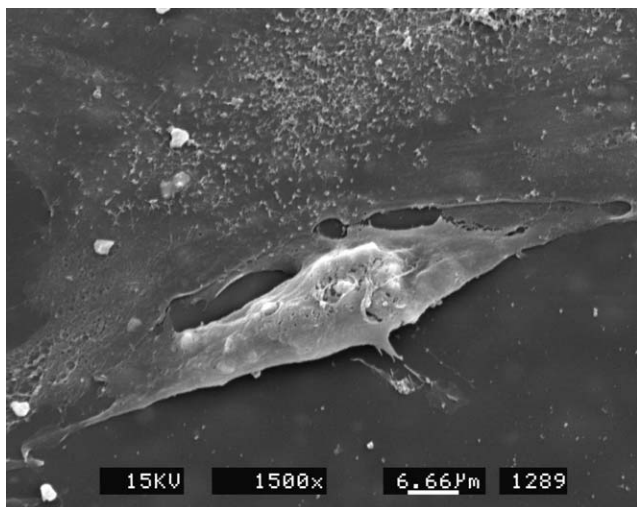


Fig. 3. Scanning electron micrographs of Schwann cells attached and adhered to the surface of GEN composite after 7 days in culture. The cells adhered and extended processes well on the membrane.

3. Results and discussions

3.1. Morphological analysis

Cell morphology on the different types of material surfaces was usually used to determine the cytocompatibility of materials [37,38]. The whole process of adhesion and spreading consists of cell attachment, filopodial growth, cytoplasmic webbing, and flattening

of the cell mass was in a sequential fashion [39]. The effect of the various membranes on Schwann cell culture was observed under the optical microscope and SEM in this study. Fig. 2 shows the morphology of the Schwann cells cultured in the control group. The cells adhered and extended processes well on the Petri dish. In addition, SEM confirmed the presence of the Schwann cells on the GEN membranes throughout the 7 days of culture. Schwann cells on GEN composites showed morphologically indistinguishable from cells plated on control dishes (Fig. 3). SEM examination also revealed that the Schwann cells flattened and extended processes in a sequential fashion as cultured for 1, 3, and 7 d.

3.2. Biocompatibility tests

The effects of different composite membranes on Schwann cell growth, as monitored by total cell protein, were shown in Fig. 4. Fig. 4 showed the effects of various membranes on the protein content of Schwann cells after 1, 3, 7 days of culture. GTG membrane with NGF immobilized by carbodiimide had constantly significant effects on Schwann cell protein content since the second day after plating. When GTG was used for Schwann cell culture with or without NGF solution soaking, it markedly decreased the cell protein content even in comparison with the control group. This result may indicate more cell attachment on GEN membrane since the protein content is higher from the second day after plating. In contrast, without immobilization of NGF, GTG membrane may even show less favorable surfaces for the attachment and growth of Schwann cells. Besides, the rate of increment in total protein content did not suggest NGF-immobilized GTG composite different from other groups. This observation

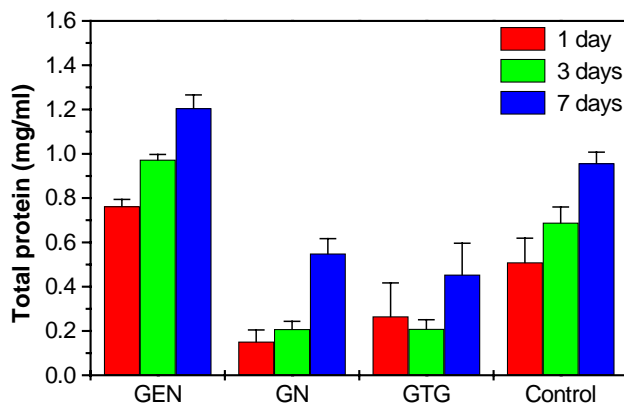


Fig. 4. Total cell protein test. It showed the effects of various membranes on the protein content of Schwann cells after 1, 3, 7 days of culture. GEN group had constantly significant effect on cell protein content since the second day after plating. GN and GTG groups markedly decreased the cell protein content even in comparison with the control group.

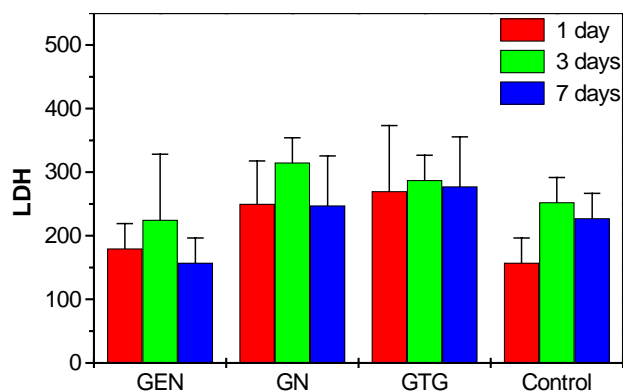


Fig. 5. LDH release test. A stable release of LDH in GTG/GN group but in a lower level in comparison with GEN/control group was noted. However, the differences in LDH leakage were not statistically significant among all the 4 groups and LDH concentration had not increased significantly from day 1 to day 7.

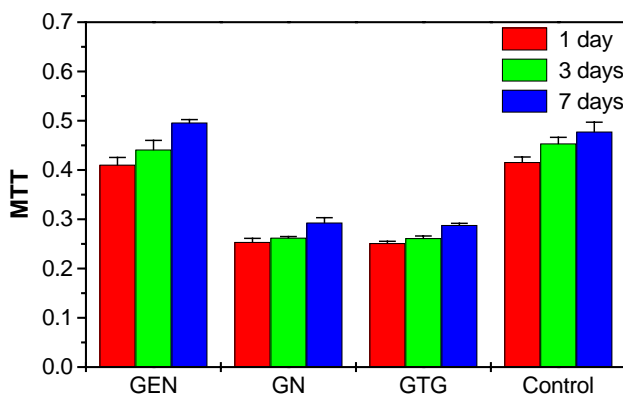


Fig. 6. MTT-tetrazolium assay. Schwann cells on the GEN membrane exhibited significantly higher total MTT reduction activity in comparison to other groups since the first day. However, for GN and GTG membrane, the MTT reduction activity of Schwann cells indicated a lower level than that on the GEN and control group.

suggests that NGF modification did not exponentially promote the growth of Schwann cells on GTG membranes.

As an intracellular enzyme, LDH was released only when cellular injury occurred. The release of LDH into culture medium provides an accurate and sensitive marker of cellular cytotoxicity in biocompatibility testing [40]. Therefore, the toxic effect of GEN/GTG composites on the cultured cells was determined by measuring the release of LDH into the culture medium. As shown in Fig. 5, a stable release of LDH in GTG/GN group but in a lower level in comparison with GEN/control group was noted. However, the differences in LDH leakage were not statistically significant among all the 4 groups and LDH concentration had not increased significantly from day 1 to day 7. This means that GTG

composite, whether cross-linked with NGF or not, has little cytotoxic effect.

The time course of Schwann cell MTT reduction activity on various membranes in each experimental group is shown in Fig. 6. The reduction of MTT mainly occurs in the mitochondria through the action of succinate dehydrogenase, therefore providing a measure of mitochondria function [41]. Schwann cells on the GEN membrane exhibited significantly higher total MTT reduction activity in comparison to other groups since the first day. Similar to the result of protein content test, there was not a rapid increasing phase of MTT reduction activity from day 1 to day 7 and the increment of MTT reduction activity in GEN group was not significantly higher than other groups. However, for GN and GTG membrane, the MTT reduction activity of Schwann cells indicated a lower level than that on the GEN and control group.

Overall, LDH assay indicates that GTG is not cytotoxic to Schwann cells. We believe that cross-linked gelatin membrane treated with glycine aqueous solution to block non-reacted aldehyde groups is important to reduce the cytotoxicity. When carbodiimide was used to immobilize NGF onto GTG membrane, the procedures do not alter the cytotoxicity of GTG membrane. The result of MTT assay also supports these observations. Comparing the protein content and MTT assay among GEN, GN composite and control group, the data confirmed more attachment of Schwann cells on the GEN composite. Thus, it indicates that the procedure used in this study to immobilize NGF onto GTG membrane is reliable. However, the time course of MTT and protein content test does not suggest that GTG grafted with NGF promote the proliferation of Schwann cells. This result is compatible with previous researches. NGF has been reported to exert no effect on new born rat Schwann cell proliferation [42,43]. In vitro, the addition of NGF into the culture medium does not alter the cell cycle nor cell morphology of Schwann cells [44]. It is not clear why NGF immobilization improves the attachment of Schwann cells on the GTG composite. However, there has been evidence suggesting that cell adhesion molecule expression on Schwann cells can be directly regulated by NGF [45]. This may partially explain the higher protein content and MTT reduction activity on GEN membranes.

In the GN group, NGF soaking procedure did not alter the attachment and growth of Schwann cells on the GTG membrane. Previously, several reports support the idea that neurotrophic factor treatment of a tubular implant facilitates nerve regeneration. With these procedures, similarly, neurotrophic factor is not covalently coupled to the surface of the biodegradable material, leaving open the possibility that neurotrophic factors desorb into solution, with the remaining concentration rapidly decreasing. From our approach,

because GTG is a biodegradable material and NGF is immobilized on to GTG covalently, the proposed mechanisms of NGF cross-linking is shown in Fig. 1. Although the release characteristics of NGF from GEN was not investigated directly in this work, the initial release of NGF could be due to hydrolysis of loosely bonded NGF. Possibly in a lower rate, covalently immobilized NGF can be released during degradation of the GEN composites. And we believe this mechanism could contribute to the sustained release of NGF. To prove the effect of NGF on neurite regeneration directly, we are now investigating the effects of various composites (GTG, GEN, GEN with cultured Schwann cells) on PC12 cells. The differentiation would be important to evaluate whether NGF or/and Schwann cells on GTG membrane promote the growth of neural cells. Furthermore, with the presence of Schwann cells, GEN membrane incorporating both cellular and acellular elements will be beneficial for axonal regeneration in peripheral nerve injury.

4. Conclusion

We report here the result of an effort to immobilize NGF onto the GTG membrane by using carbodiimide. The experiments carried out on the effect of NGF grafted onto GTG membranes indicate that the procedure used in immobilization of NGF onto GTG membrane may be beneficial for adhesion of Schwann cells on the membranes. Another finding of this study is the feasibility of culturing Schwann cells on GEN and GTG membranes. None of the materials (GEN/GN/GTG) appeared to cause any alteration in Schwann cell morphology. Without immobilization of NGF on the surfaces, GTG membrane still showed cytocompatibility with Schwann cells. With the techniques used in this study, we will be able to design a tissue-engineered biodegradable material that incorporates with Schwann cells and covalently immobilized NGF. During the degradation of GEN composite, there is the potential for sustained release of NGF. Therefore, it is reasonable to make use of GEN membrane to improve axonal regeneration of injured peripheral nerve. A thorough study of the effects of Schwann cell seeded GEN membrane on the growth of neurons and animal experiments will be followed in the future.

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