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Controlled Release of Nerve Growth Factor from Sandwiched Poly(L-lactide-*co*-glycolide) Films for the Application in Neural Tissue Engineering

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Abstract: In order to fabricate new sustained delivery device of nerve growth factor (NGF), we developed NGF-loaded biodegradable poly(L-lactide-*co*-glycolide) (PLGA, the mole ratio of lactide to glycolide 75:25, molecular weight: 83,000 and 43,000 g/mole, respectively) film by novel and simple sandwich solvent casting method for the possibility of the application of neural tissue engineering. PLGA was copolymerized by direct condensation reaction and the molecular weight was controlled by reaction time. Released behavior of NGF from NGF-loaded films was characterized by enzyme linked immunosorbent assay (ELISA) and degradation characteristics were observed by scanning electron microscopy (SEM) and gel permeation chromatography (GPC). The bioactivity of released NGF was identified using a rat pheochromocytoma (PC-12) cell based bioassay. The release of NGF from the NGF-loaded PLGA films was prolonged over 35 days with zero-order rate of 0.5~0.8 ng NGF/day without initial burst and could be controlled by the variations of molecular weight and NGF loading amount. After 7 days NGF released in phosphate buffered saline and PC-12 cell cultured on the NGF-loaded PLGA film for 3 days. The released NGF stimulated neurite sprouting in cultured PC-12 cells, that is to say, the remained NGF in the NGF/PLGA film at 37 °C for 7 days was still bioactive. This study suggested that NGF-loaded PLGA sandwich film is released the desired period in delivery system and useful neuronal growth culture as nerve contact guidance tube for the application of neural tissue engineering.

Keywords: NGF, PLGA, film, sandwich solvent casting method, NGF release, PC-12 cell.

Introduction

Neurodegenerative diseases, such as Alzheimers disease and Parkins disease and traumatic injury to the central nervous system (CNS), such as in spinal cord injury (SCI), result in axonal degeneration, neuronal necrosis, gliosis, and proliferation of reactive astrocytes. After SCI, patients are left paralyzed with only palliative measures available but no therapy to restore function.[1,2]

Very recently, it has been recognized that tissue engineering is starting to offer an alternative techniques to SCI. To reconstruct a central nerve system, cells which is harvested and dissociated from the donor nerve cells organ and scaffold substrates such as nerve guidance channel which cells are attached and induced resulting in the implantation at the axon of the functioning tissue must be needed.[3-5] Another important factor for the regeneration of CNS is neurotrophic factors. Among a large variety of neurotrophic factors, the nerve growth factor (NGF) has been characterized to promote both the growth and survival axon of the peripheral and CNS, and the survival and differentiation of transplanted neural tissue as well as the neuropathy of Alzheimers disease and the potential treatment for brain.[6-8] However, one of the significant problems of the administration of protein drug is a short half-life in plasma and poor penetration at the blood barrier.[2,8] Hence, it is strongly wanted to develop new delivery method. Several new methods for the controlled delivery of

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NGF to the CNS and brain have been suggested and widely tested such as (1) controlled release from polymer matrices with NGF, (2) controlled release from biodegradable NGF-loaded microspheres, and (3) controlled delivery by cells that were genetically modified to produce NGF.[9,10]

Recently, the family of poly(α-hydroxy acid)s such as polyglycolide (PGA), polylactide (PLA) and its copolymer like poly(lactide-*co*-glycolide) (PLGA) which are among the few synthetic polymers approved for human clinical use by Food and Drug Admnistration is extensively used or tested for the scaffolds materials as a bioerodible material due to good biocompatibility, controllable biodegradability, and relatively good processability.[11] These polymers degrade by nonspecific hydrolytic scission of their ester bonds.[12] The hydrolysis of PLA yields lactic acid which is a normal byproduct of anaerobic metabolism in human body and is incorporated in the tricarboxylic acid (TCA) cycle to be finally excreted by the body as carbon dioxide and water. PGA biodegrades by a combination of hydrolytic scission and enzymatic (esterase) action producing glycolic acid[13] which can either enter the TCA cycle or be excreted in urine and be eliminated as carbon dioxide and water.[14] The degradation time of PLGA can be controlled from weeks to over a year by varying the ratio of monomers and the processing conditions.[15] It might be a suitable biomaterial for use in tissue engineered repair systems[16-26] in which cells are implanted within PLGA films or scaffolds and in drug delivery systems[27-35] in which drugs are loaded within PLGA microspheres.

In our previous study,[9,10,36] we studied NGF-loaded PLGA devices such as microspheres and scaffolds for the possibility of the application of neural tissue engineering. One of the significant disadvantages is the massive initial burst from the surface of NGF loaded microspheres and interconnected pores of the PLGA scaffolds and the massive loss during the preparation process resulting in no detection of accurate NGF concentration. In this study, in order to overcome these problems, we developed the novel and simple preparation method for NGF-loaded PLGA film such as sandwich method, especially, for the possibility of the nerve guidance channel. PLGA was synthesized by direct melt polymerization. Released behavior of NGF from NGF-loaded films was characterized by enzyme linked immunosorbent assay (ELISA) and degradation characteristics were observed by scanning electron microscopy (SEM) and gel permeation chromatography (GPC). Also the bioactivity of released NGF was identified using a rat pheochromocytoma (PC-12) cell based bioassay.

Experimental

Materials. L-lactide and glycolide monomers were purchased from Boehringer Ingelheim (Ingelheim, Germany). Initiator as stannous octoate was purchased from Wako Chem. Co., (Osaka, Japan). Toluene (Jusei Chem. Co. Tokyo, Japan) and methylene chloride (MC, Tedia Co. Inc., USA) were used as received. All other chemicals were a reagent grade. NGF (molecular weight: 26,000 g/mol, NGF-7S, Sigma Chem. Co., St. Louis, MO, USA) and NGF-ELISA kit [Anti-β (2.5S, 7S) Nerve Growth Factor, mouse and Anti-β (2.5S, 7S) Growth Factor-β-gal, mouse Boelinger-Manheim, Germany] were used as received.

PLGA Synthesis and Characterization. A 30 g mixture of the L-lactide (75 mole %) and glycolide (25 mole %) was preheated in an evacuated flask at 60 °C for 2 hrs to remove water trace. Stannous 2-ethylhexanoate in toluene (150 ppm) was added to polymerization reactor (ϕ30 mm × 35 cm length) with the agitation of 100 rpm. Dry nitrogen gas was flushed through whole processing. After adding the catalyst, the copolymerization reaction was carried out at 165 °C for 2 and 4.5 hrs, respectively. The light brownish PLGA obtained was purified by dissolving in MC, followed by slow precipitation in excess methanol. The polymer was dried *in vacuo* at room temperature for 7 days and kept until use. Further detailed procedures for copolymerization were described in previous papers.[9,10,16,17]

To characterize synthesized PLGA, GPC was performed. Measurement was carried out on a Waters Chromatograph 200 Series equipped with μ-Styragel® columns in series with 10^5, 10^4, 10^3, and 500 Å of pore size, respectively. Tetrahydrofuran was used as an eluent solvent. The temperature, the flow rate, and the injection volume were 30 °C, 1 mL/min, and 15 μL, respectively. The series of polystyrene monodisperse standard were used to calibrate the molecular weight. The average molecular weight (MW) and molecular weight distribution (MWD) of purified samples with 2 and 4.5 hrs of reaction were around 43,000 g/mole and 1.90 and 83,000 g/mole and 1.84, respectively, with relatively good reproducibility.

Preparation of NGF-loaded PLGA Films by Sandwich Method. A schematic diagram of the fabrication process by sandwich method is shown in Figure 1. First, 0.3 g of PLGA was dissolved in 6 mL of MC. PLGA solution was cast onto Pyrex glass dish (ϕ50 mm; 19.63 cm^2 area) on a horizontal level in order to get 200-300 μm thickness of PLGA films. After evaporation of MC at room temperature, 500 ng and 1 μg of NGF in 2 mL deionized water was poured onto PLGA films, then freeze dried at -56 °C using freeze dryer (FD5508, Ilshin Sci., Co. Ltd., Korea). These NGF amount were equivalent to 25.4 and 50.9 ng/cm^2, respectively. Again, same concentration amount of PLGA solution was cast onto NGF layer, that is to say, NGF layer was sandwiched between PLGA layer (total thickness; 500 μm). After evaporation of MC for 2 days at room temperature, NGF-loaded films were freeze-dried for 2 days to eliminate remained water and solvent. All samples were kept in a vacuum oven until use.

Determination of NGF Release Amount from NGF-loaded PLGA Film. Appropriate amount of NGF-loaded

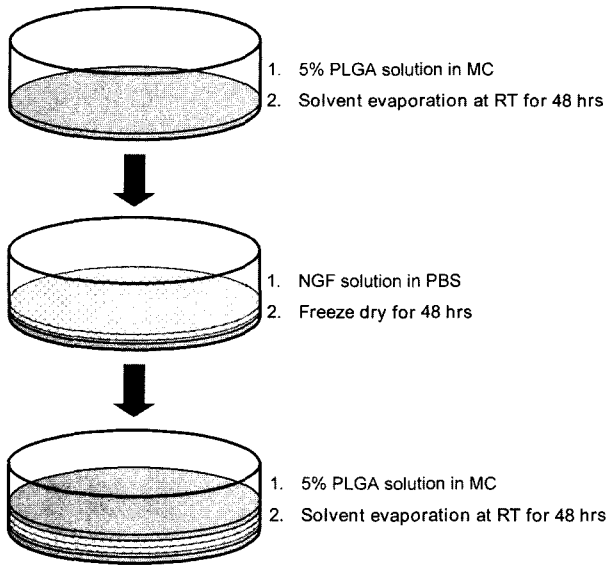

Figure 1. Schematic diagram of preparation of NGF-loaded PLGA films by sandwiched solvent casting method.

PLGA film (1×1 cm^2) was placed in phosphate buffered saline solution (PBS, pH: 7.4, Sigma Chem. Co.) at 37 °C. Then NGF released aqueous solution was taken at scheduled time and refilled fresh PBS with same taken amount. The amount of NGF was determined by ELISA kit and calibrated by Microplate Reader (Thermolex, Molecular Device Co. Ltd., USA).[1,9,10]

Investigation of Biodegradation. First, changes MW of PLGA were analyzed by GPC at scheduled time as described earlier. Second, surface and cross sectional morphology of NGF loaded PLGA film were observed by SEM (Model S-2250N, Hitachi Co. Ltd., Japan) to investigate the biodegradation behavior. Samples sliced by sharp razor for SEM were mounted on metal stub with double-sided tape and coated with platinum for 30 sec under argon atmosphere using plasma sputter (SC 500K, Emscope, UK).

Bioassay of Released NGF. PC-12 cells (KCLB 21721, Korea Cell Line Bank, Korea) were cultured in Dulbecco's modified Eagles medium (DMEM, GIBCO BRL Co., USA) with L-glutamine, supplemented with 15% in activated donor calf serum (CS, GIBCO BRL) and antibiotic/antimycotic (penicillin 10,000 U/mL, streptomycin 10,000 cgm/mL, and amphotericin B 25 μg/mL, GIBCO BRL) in tissue culture polystyrene flask (Falcon, Co., USA) at 37 °C under 5% CO_2 atmosphere.[9,10,36] To observe NGF bioactivity after release of NGF at scheduled time, PC-12 cells were plated at a density of 8×10^4 cells/cm^2 onto the NGF-loaded PLGA films eliminated with NGF released aqueous solution in 12-well plate for 7 days. After incubation at 37 °C under 5% CO_2 atmosphere, the PLGA surfaces were washed with PBS and the PC-12 cells attached on the surfaces were fixed with 2.5% glutaraldehyde (GIBCO BLR) in PBS for 24 hrs at room temperature. After thorough washing with PBS, the cells on the surfaces were dehydrated in ethanol graded series (50, 60, 70, 80, 90, and 100%) for 10 min each and allowed to dry in a clean bench at room temperature. The cell-attached PLGA surfaces were gold deposited in vacuum using plasma sputter and examined by SEM with a tilt angle of 45 degree for observing cell morphology.

Results and Discussion

It has been recognized that NGF absolutely stimulates the development and growth of adrenal medullary tissue and promotes neuronal-like transformation of adrenal chromaffin cell. Also, it has already been demonstrated that adrenal medullary cells survived in great numbers in 6-OH dopamine-treated rats when supplied with NGF and that functional recovery improved. To overcome the drawback of conventional NGF administration methods such as injections, minipumps and transplanted cells engineered to produce NGF, we prepared NGF-loaded PLGA biodegradable film by means of the sandwich solvent casting for the possibility of the application of tissue engineered nerve regeneration.

Degradation of NGF-loaded PLGA Films. NGF-loaded PLGA film was clear and plain. PLGA films were immersed in PBS at 37 °C, maintained at pH 7.4 and characterized for 35 days in terms of changes in MW and MWD by GPC and in morphology by SEM. As listed in Table I, PLGA steeply degraded to ~30% of initial MW within 7 days and almost completely to 2,000 g/mole after 21 days with film state. It was also observed that MWD decreased from 1.8~2.0 to around 1.2 within 21 days due to even chain scission. The degradation morphology of PLGA films was confirmed by SEM as shown in Figure 2. We could observe a cross section of very thin sandwiched NGF layer in Figure 2(A) (arrow a) in initial. As shown in Figure 2(B), after 3 days of incubation in PBS, many pores can be investigated in the cross section

Table I. Changes of Molecular Weight and Molecular Weight Distribution of NGF-loaded PLGA Films by GPC

Time (days)	1	7	14	21	28
MW (g/mole)	83,000	27,000	15,000	1,900	2,000
MWD	1.84	1.74	2.04	1.28	1.25
MW (g/mole)	43,000	20,000	12,000	2,000	2,000
MWD	1.90	1.76	2.00	1.20	1.18

likely resulting from the leaching out of water soluble degradation products as lactide and glycolide oligomers. It can be observed much more and bigger pores generated for 7 days. However, surface of NGF-loaded PLGA film was still plain. It can be explained that the degradation mechanism of poly(α-hydroxyester) appears bulk erosion attributed to the autocatalytic hydrolytic degradation mechanism where released carboxylic acids contribute to an increased rate of hydrolysis along the polymer backbone rather than surface erosion.[37]

Release Profiles of NGF. The release profile of NGF from NGF-loaded PLGA film over time was investigated with different PLGA molecular weights as 43,000 and 83,000 g/mole and different loading amounts as 25.4 and 50.9 ng/cm^2 by ELISA method. Cumulative amount of released NGF and logarithmic plot of release rate of 43,000 g/mole PLGA showed in Figures 3(A) and (B), respectively. Initially, for 50.9 ng/cm^2 NGF loading, NGF did not release to day 3, then released to zero order rate from day 3, that is to say, NGF released 0.1 ng/day from 0 to ~3 days then release rate increased to 1 ng/day from ~7 days. This increased release rate can be explained by rapid degradation phenomena in Figure 2 such as the formation of porous structures. This release rate kept almost 0.8 ng/day over 35 days continuously. Also, the NGF release profile increased with increasing NGF loading amount.

Cumulative amount of released NGF and logarithmic plot of release rate of 83,000 g/mole PLGA showed in Figures 4(A) and (B), respectively. Behavior of NGF release pattern was almost same trends with those of 43,000 g/mole PLGA. The release rate kept 0.5~0.6 ng NGF/day in which was

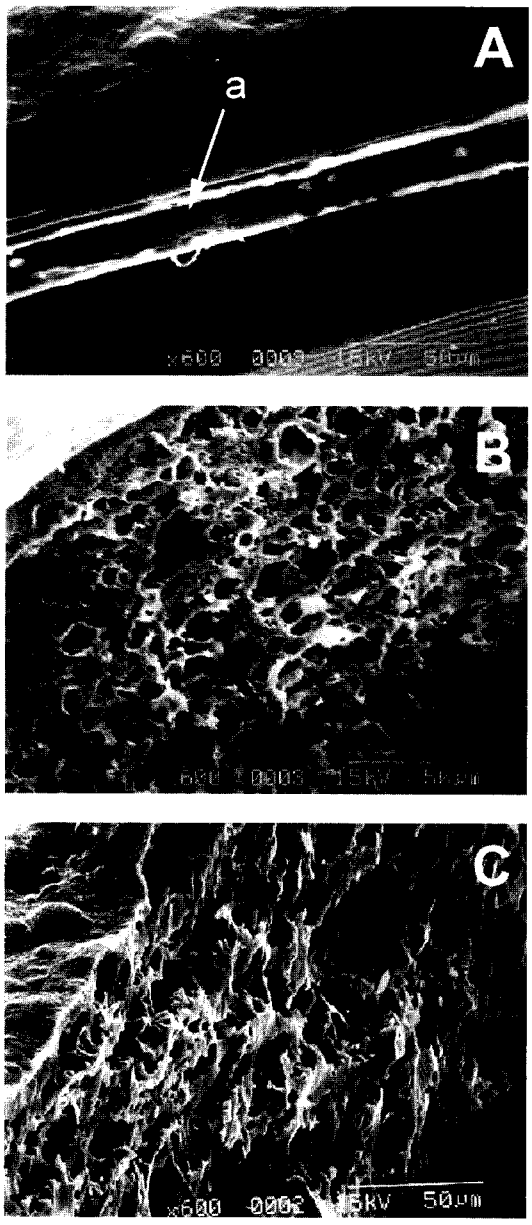

Figure 2. SEM pictures of degradation morphologies of NGF-loaded PLGA films after (A) 0, (B) 3, and (C) 7 days. (molecular weight of PLGA; 83,000 g/mole, original magnification ; × 600).

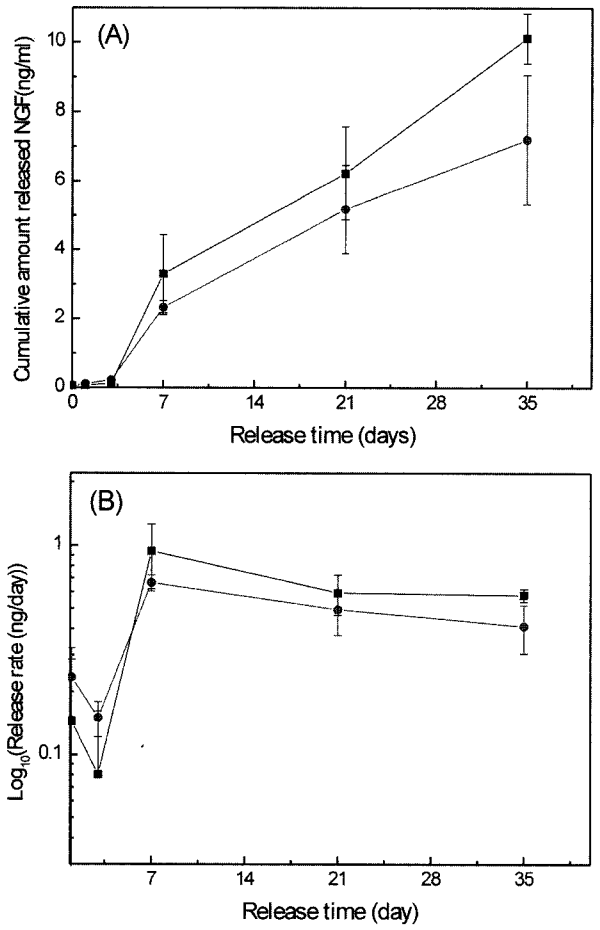

Figure 3. (A) Release profiles and (B) logarithmic plot of release rate for NGF from NGF-loaded PLGA films of 43,000 g/mole. (●) 25.4 ng and (■) 50.9 ng NGF/cm^2 PLGA.

lower than that of 43,000 g/mole PLGA over 35 days. Compared with Figures 3(A) and 4(A), cumulative NGF released amount of 43,000 g/mole PLGA was little bit higher than that of 83,000 g/mole PLGA due to the faster biodegradation rate resulting in easier formation of oligomer.

According to the numerous previous studies for the protein sustained delivery devices using PLGA system such as microspheres and microporous scaffolds including our works,[1,2,8-10,37] massive initial burst of protein drug as vascular endothelial growth factor and NGF must be observed, even though the rate of initial burst was somewhat different. However, the initial burst of NGF could not observe NGF-loaded PLGA film. In the case of microspheres and microporous scaffolds, initial burst phenomena came from massive dissolution of the adsorbed protein on the surface of microspheres and the interconnected surfaces between the pores during the microencapsulation process and foaming process, respectively, whereas this sandwiched method did not formed the NGF adsorbed PLGA surfaces as well as any loss of protein cytokines. The protein release kinetics for PLGA microspheres and microporous scaffolds are following; (i) initial release phase of adsorbed proteins, (ii) PLGA degradation, (iii) protein aggregation and/or protein adsorption, and (iv) PLGA/protein interaction and/or entanglement. Sometimes, phases of (iii) and (iv) caused to pulsed release of proteins as well as low molecular weight drug.[38,39] Therefore their relative influences were never clearly identified and several phenomena coexisted.

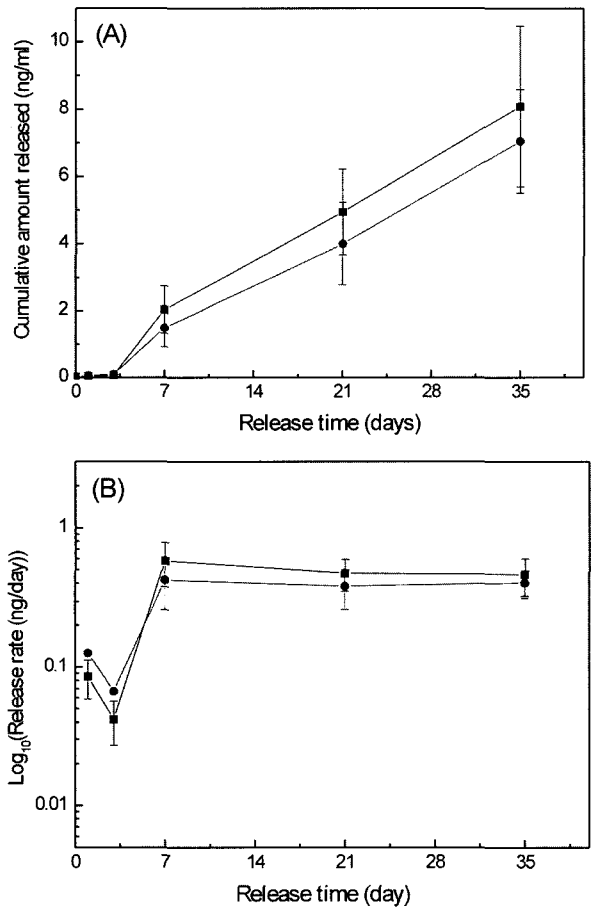

Figure 4. (A) Release profiles and (B) logarithmic plot of release rate for NGF from NGF-loaded PLGA films of 83,000 g/mole. (●) 25.4 ng and (■) 50.9 ng NGF/cm^2 PLGA.

In our case, first no NGF release investigated within 3 days, second pulsed release observed days 3 in both molecular weights just after massive PLGA biodegradation, and then almost constant release rate kept for days 7 to 35. In summary, from the results of release pattern and degradation behavior in our device, the release kinetics of NGF sandwiched PLGA film might be suggested as follows; (i) PLGA degradation, (ii) NGF aggregation/adsorption with PLGA oligomer, (iii) solubilization or diffusion of NGF or PLGA/NGF complex, then (iv) NGF dissociation without any diffusion of NGF across PLGA bulk.[40]

Bioactivity of Released NGF. In order to assure the bioactivity of releasing NGF from the NGF sandwiched PLGA film, PC-12 cell as a model cell line originated from pheochromocytoma in the rat adrenal medulla cultured onto the PLGA film after 7 days release. One of the important features of PC-12 cells is that they respond to NGF. PC-12 cells can be induced to differentiate into cells with typical neuron morphology by NGF.[36] After several days exposure to NGF, PC-12 cells undergo dramatic change in phenotype and acquire a number of properties characteristics of sympathetic neurons, including the extension of neurites. For instance, the NGF-treated cells cease proliferation, grow long neurites, become electrically excitable and show a number of changes in composition associated with enhanced neuronal differentiation. To generate neurites PC-12 cells are plated at the above density in the presence of 50 ng/mL (~2 mM) NGF. Neurite induction of the PC-12 cell is also an important research area for nerve regeneration.

Figure 5 shows the regeneration of neurites of PC-12 cells for 3 days cultivation which were plated onto (A) PLGA, (B) 25.4 ng NGF/cm^2 PLGA and (C) 50.9 ng NGF/cm^2 PLGA of 83,000 g/mole just after 7 days released, respectively. We could not observe any neurites on control PLGA whereas significant growth of neurites was observed on NGF/PLGA film. The higher concentration of NGF, the longer neurites protruded. Figure 6 shows the generation of neurites of PC-12 cells in which were plated at the density in the presence of 0, 10, and 50 ng/mL NGF on the control polymeric surfaces, respectively. It was observed that PC-12 cells did not generate neurites in the presence of 0 and 10 ng/mL NGF whereas PC-12 cells generated neurites at 50 ng/mL NGF. Thus, to generate neurites, PC-12 needed a density of 50 ng/mL NGF as discussed above, that is the biological activity of NGF depends on concentration at the site of action. Even though our NGF sustained delivery system was quite different from aqueous NGF culture system

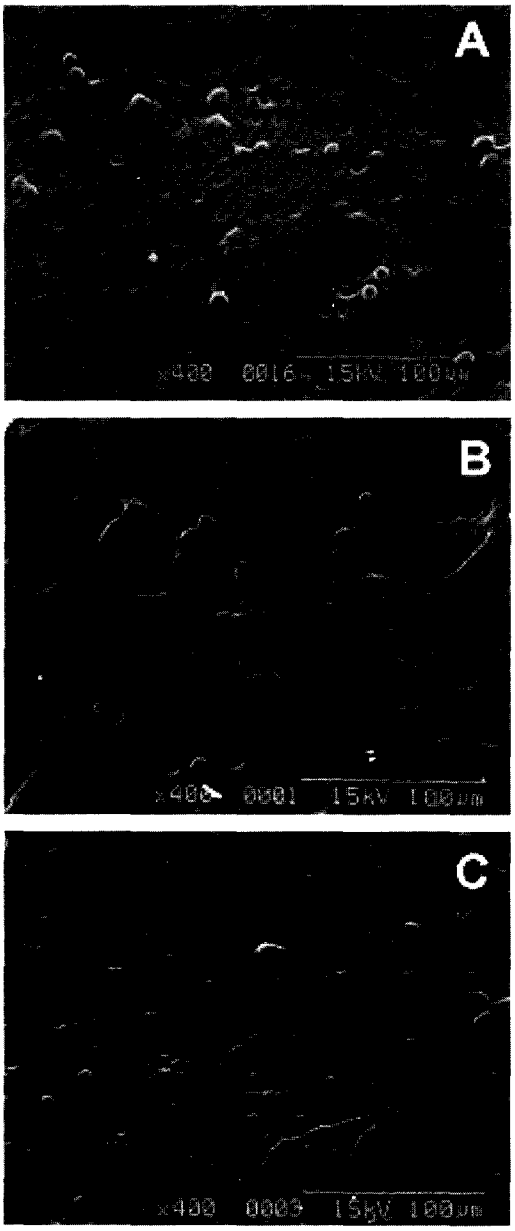

Figure 5. Effect of NGF concentration on neurites formation of PC-12 cells for 3 days cultivation on control: (A) PLGA, (B) 25.4 ng, and (C) 50.9 ng NGF/cm^2 PLGA just after 7 days. (molecular weight of PLGA; 83,000 g/mole, original magnification ; × 400).

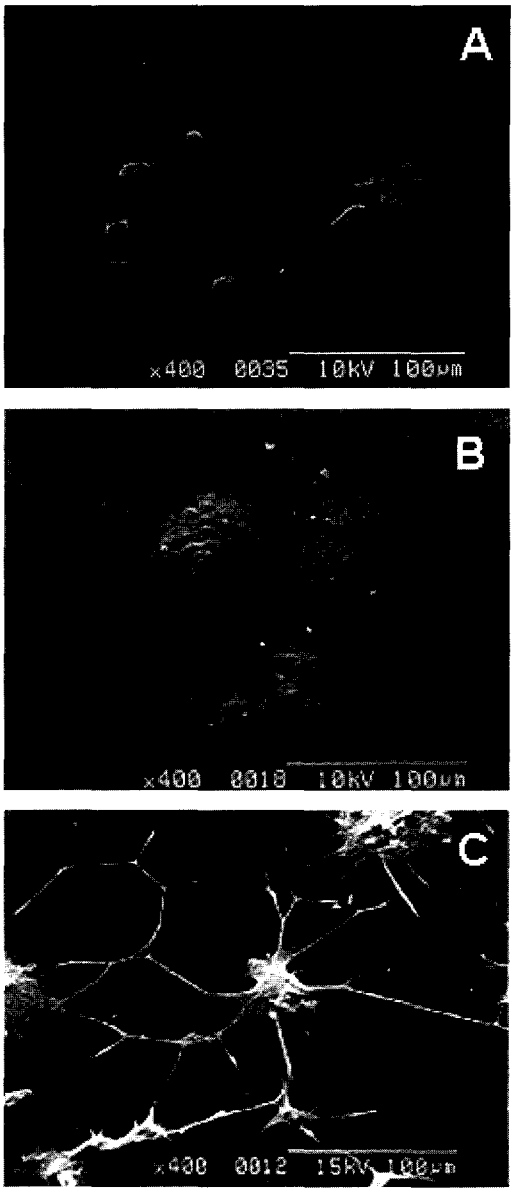

Figure 6. SEM pictures of the PC-12 cell cultured with (A) 0, (B) 10, and (C) 50 ng/mL NGF after 4 days culture. (original magnification; × 400).

as Figure 6, that is, NGF concentration in culture media of NGF/PLGA system was quite lower than aqueous system, biological activity of NGF for our system was still existed. One of significant disadvantages of conventional administration of cytokines is the short half-life below 12 hrs in plasma.[2,8-10] Thus this proposed method might be very useful for the prolonged cytokine administration at desired site, especially, nerve guidance channel or depot of neurotorpic factor for the treatment of central nervous injury and Alzheimer or Parkinson's disease of brain, respectively. Also, typical advantage of this proposed study is very simple fabrication method compared microspheres and microporous scaffold as well as relative very short contact time with solvent and water resulting in decrease of the possibility of denaturation of protein drug.

Generally speaking, the physical and chemical requirements of scaffolds for the application for tissue engineering are[41,42] (i) biocompatibility, (ii) promotion of cell adhesion, (iii) enhancement of cell growth, (iv) retention of differentiated cell function, (v) large surface area per volume, (vi) highly porosity to provide adequate space for cell seeding, growth

and extracellular matrix production, and (vii) a uniformly distributed and interconnected pore structure (this factor is very important so that cells are easily distributed through the scaffolds and an organized network of tissue constituents can be formed). For the case of nerve conduit channel for the application of nerve tissue engineering, it can be suggested that pore structure must not be needed because only conduit act the guidance for the regeneration of axon from proximal to distal direction, the prevent of any harmful biological molecules nearby regeneration site and the supply of neurotropic or nutrient factor continuously.

Studies on the more detailed release mechanism of NGF from NGF/PLGA films, bioactivity (same meaning as extent of denaturation) of NGF during manufacturing process, the biodegradation test with different molecular weight and different dimension, the optimized fabrication method of tubular of NGF sandwiched PLGA films, the implantation this device with mesenchymal stem cell, the optimal diameter and length of tubular for the application of tissue engineered nerve, and its relative animal experiment are in progress.

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