J. Microbiol. Biotechnol. (2009), **19**(11), 1490–1495
doi: 10.4014/jmb.0903.03002
First published online 18 July 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Neuronal Differentiation of PC12 Cells Cultured on Growth Factor-Loaded Nanoparticles Coated on PLGA Microspheres

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Received: March 10, 2009 / Revised: May 10, 2009 / Accepted: June 2, 2009

The development of nanotechnology has penetrated the fields of biology and medicine, resulting in remarkable applications for tissue regeneration. In order to apply this technology to tissue engineering, we have developed nano-scaled 3D scaffolds consisting of growth factor-loaded heparin/poly(l-lysine) nanoparticles (NPs) attached to the surface of polymeric microspheres *via* polyionic complex methods. Growth factor-loaded NPs were simply produced as polyelectrolyte complexes with diameters of 100–200 nm. They were then coated onto positively charged poly(lactic-co-glycolic acid) (PLGA) pretreated with polyethyleneimine to enable cell adhesion, proliferation, and stimulation of neurite outgrowth. Propidium iodide staining and β-tubulin analysis revealed that neuronal PC12 cells proliferated extensively, expressed significant amounts of b-tubulin, and showed well-structured neurite outgrowth on polymeric microspheres by stimulation with growth factors. These results suggest that cellular adhesion and biological functionality on prepared PLGA microspheres enabled terminal differentiation of neuronal cells.

Keywords: Nanotechnology, tissue regeneration, 3D scaffolds, polyethyleneimine, PC12 cells

In recent decades, researchers have focused on clinical attempts to regenerate damaged nerve tissues that involve implanting nerve cells or grafting them toward the defect site [7, 8, 10, 20, 23]. Although engineering of ideal nerve regeneration methods offers fascinating prospects, many shortcomings remain, such as the necessity of complex surgical procedures and loss of function in the defect site [6, 11, 21]. In order to overcome these problems, control of implanted cell functions must be regulated for differentiation and neurite outgrowth; hence, the development of suitable scaffolding materials to support cells on implantation is required [1, 5, 22]. The materials must provide appropriate chemical and spatial micro- or macroenvironments for cell proliferation, differentiation, and neurite outgrowth. Recently, we have begun to see the emergence of applications of nanotechnology in neuroscience using primarily organic and polymeric materials as scaffolds. Some research has shown that neural stem cells and PC12 cells can be successfully differentiated to neurons with clear formation of neurites on layer-by-layer (LBL) assembled single-walled carbon nanotube (SWNT)-polyelectrolyte multilayer thin films and hydrogel scaffolds [2, 4, 9, 16]. In previous studies, we reported the fabrication of PLGA microspheres coated with dispersed NPs as a vehicle for a host of applications, including delivery of stem cells and proteins or peptides [13, 15].

Here, we focus on the development of a new platform biomaterial based on PLGA microspheres that can find applications in the treatment of neurological disorders and injuries. PLGA microspheres could play the important role of microscale scaffold in tissue engineering, providing a microenvironment site for delivery of cells or proteins in desired locations in the body and defining a three-dimensional space for the formation of new nerve tissues. As the PLGA microspheres degrade, the microsphere spaces could be replaced with newly regenerated tissues [3, 17].

During neurite outgrowth in a regenerating nerve, growth factor will be released by the nerve growth cone *via* proteolysis, enabling its use by that very neurite [18]. In addition, association of the growth facter with matrix-binding sites could help to maintain its bioactivity[18].

Heparin chains, being highly negatively charged, easily bind to angiogenesis growth factors to complex a stable form that supports biological activity and can retard release [7, 13]. In this report, we show that growth factor immobilized in heparin complexed with PLL is an adequate

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material for *in vitro* nerve cell culture. In addition, in order to determine the biological effects that can be triggered in nerve cells specifically by growth factor loaded on NPs attached to microspheres, PLGA microspheres alone, NP-coated microspheres, and basic fibroblast growth factor (bFGF)-loaded NP-coated microspheres were used as matrices for culture and differentiation of PC12 cells. In doing so, we demonstrate that application of a biological stimulus through growth factor-loaded heparin/PLL NPs enhances neurite outgrowth.

MATERIALS AND METHODS

Materials

Low-molecular-weight heparin (LMWH, Fraxiparin), which has an average molecular mass of approximately 4,500 Da, was obtained from Sanofi-Synthelabo Co. (Gentilly, France). PLL (MW=3,000–7,000 Da) was purchased from Sigma-Aldrich. PLGA with a molecular weight of 33,000 and a copolymer ratio of lactide to glycolide of 50:50 (R503H, Boehringer Ingelheim, Germany) was used as wall material for the microspheres. All reagents and organic solvents used were of at least ACS grade.

Preparation of Heparin/PLL Nanoparticles

All materials were obtained from commercial sources, and were used without additional purification. Specific amounts of a concentrated heparin solution (0.5 mg/ml) were added to a solution of PLL (0.5 mg/ml) in distilled water, yielding complexes with −/+ molar charge ratios in a range from 0 to 2.95. The density of charges on heparin was assumed to be 5 mEq/g. Dynamic light scattering (DLS) analyses were conducted after each incremental addition of heparin. Both stock heparin and polymer solutions were passed through 0.22-μm nylon filters prior to experimentation.

Preparation of PLGA Microspheres

PLGA microspheres were prepared using solvent evaporation in an oil-in-water emulsion. Four of PLGA was dissolved in 30 ml of dichloromethane. Using a glass syringe and a 20-gauge needle, the polymer solution was dropped into 300 ml of aqueous solution containing 2% (w/v) polyvinyl alcohol (PVA) while mixing, using a magnetic stirrer at 600 rpm. The suspension was then gently stirred for 2 to 3 h at 35°C with a magnetic stirrer at 600 rpm in order to evaporate the dichloromethane. Microspheres were collected *via* 2 min of centrifugation at 1,500 rpm, washed four times in distilled water, and then lyophilized. Microsphere size, as measured by SEM, ranged between 20 and 80 μm.

Immobilization of Growth Factor-Loaded Heparin/PLL Nanoparticles on PLGA Microspheres

First, the PLGA microspheres were coated with positively charged polyethyleneimine (PEI) at the native pH (7.4) of the polymer solution with no additional salt. Under these conditions, the PEI side chain amines (pKa≈10) would be extensively protonated. The microspheres (1 g) were soaked for 12 h in the PEI solution (1 mg/ml) with gentle stirring, collected through 2 min of centrifugation at 1,500 rpm, rinsed three times in distilled water, and then dipped and soaked in heparin/PLL nanoparticles loaded with growth factor solution (1 mg/ml) for 24 h with gentle stirring. The PLGA microspheres were then collected *via* 2 min of centrifugation at 1,000 rpm and rinsed three times in distilled water. Finally, the microspheres were washed four times in distilled water and lyophilized.

Growth Factor Release from PLGA Microspheres

One hundred mg of microspheres coated with bFGF (100 ng/ml) in a dialysis bag was evaluated for release characteristics. Aliquots were relocated to conical polypropylene tubes containing 1 ml of minimal essential medium (α-MEM), and placed on an orbital shaker. The medium was removed, frozen, and replenished on days 1, 3, 5, 7, 10, 14, 21, and 28. Enzyme-linked immunosorbent assay (ELISA) for bFGF was performed to determine the concentration of immunoreactive protein. Blank PLGA microspheres were used as controls.

Cell Seeding and Growth on Heparin-Bound bFGF-Coated Polymeric Microspheres

PC12 cells were cultured in 25-cm^2 flasks containing 10 ml of RPMI 1640 medium supplemented with HEPES and L-glutamate, 10% horse serum, 5% fetal bovine serum, and standard penicillin–streptomycin at 37°C under 5% CO_2 and 95% air. Culture medium was exchanged every 3–4 days and subculture was performed approximately once every two weeks at a ratio of 1:3 and 1:5, using 0.5 ml of 0.125% trypsin in citrate saline. For the cell seeding and growth test, 100 mg of microspheres and PC12 cells at 5×10^5 cells/ml were incubated in a Transwell insert in a culture dish with gentle shaking. After 2 h, unattached cells were removed and the insert was incubated further for cell growth.

Scanning Electron Microscopy (SEM) Analysis

Scanning electron microscopy (Philips 535M) was used to observe the size and morphology of PC12 neurites. Morphology was observed after gold coating using a sputter-coater (HUMMER V, Technics, CA, U.S.A.). Argon gas pressure was set at 5 psi and the current was maintained at 10 mA for 5 min. For observing the morphology of cells attached to PLGA microspheres, the cells were treated with 2.5% (v/v) glutaraldehyde in PBS, fixed at 4°C overnight, rinsed with warm PBS for 5 min, and immersed for 1 h in 1% (w/v) osmium tetroxide dissolved in 0.1 M sodium cacodylate. The samples were washed twice with deionized distilled water and dehydrated through a graded ethanol series (25%, 50%, 75%, and 90%) for 5 min each, and then washed three times with 100% ethanol for 10 min. The ethanol was completely evaporated by air flow in a clean bench before gold coating.

B-Tubulin Assay of PC12 Cells

Briefly, stock solutions of 1 mg/ml propidium iodide (PI) were diluted with distilled water to 2% (v/v). Six μl of each was added to 200 μl of Vectashield (Vector Laboratories, CA, U.S.A.) on a slide, to give a final concentration of approximately 0.6 μg/ml PI. A coverslip was applied and the edges were sealed with rubber solution (Dunlop Adhesives, Birmingham, U.K.). Slides were examined using a Nikon Microphot SA microscope equipped with a Nikon Chroma fluorescence filter. Some preparations were also examined using a Nikon E600 microscope with independent PI and signal integration software (Applied Imaging International Ltd., Newcastle Upon Tyne, U.K.). Tubulin was localized by incubation with fluorescein-conjugated monoclonal antitubulin antibody (clone DM1A; Sigma-Aldrich),

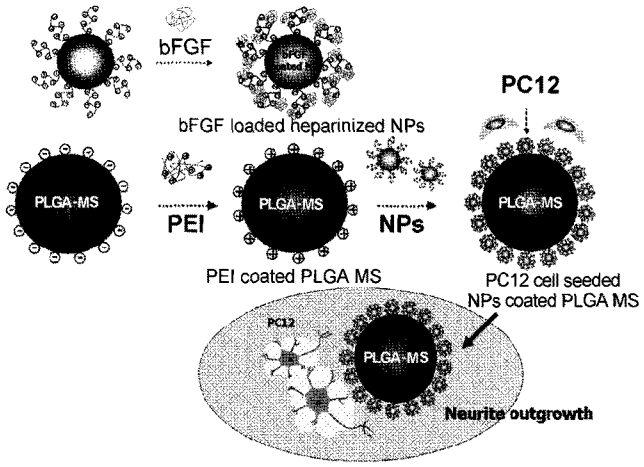

Fig. 1. Schematic diagram of bFGF-loaded heparinized nanoparticles coated on a PLGA microsphere for stimulating neurite outgrowth.

diluted 1:100 in PBS, for 1 h a 37°C. Nuclei staining was performed by incubation with PI for 15 min at room temperature. Coverslips were mounted in Mowiol (Calbiochem) DABCO (Sigma-Aldrich) and slides were examined under a fluorescence microscope.

Results and Discussion

Fig. 2 shows negatively charged polyplexed NPs, consisting of heparin and PLL and carrying bFGF, immobilized and coated onto PLGA microspheres by pretreatment with the cationic polymer PEI. Several images show that all regions of the microsphere surface, including middle, upper, and lower regions, were coated with NPs (Fig. 2B–2F). Owing to their high binding potential, the NPs were highly immobilized and well distributed on the PLGA microspheres, which were well coated. The outer part of the polyplexed NPs, the negative charge-carrying heparin residues complexed with PLL, are not reduced as a result of the immobilization procedure. In a previous study, we evaluated the immobilization of polyplexed NPs on PLGA microspheres for chondrogenesis of attached mesenchymal stem cells [13]. In this study, heparinized NPs not loaded with growth factors showed a much larger size (300–500 nm) compared with growth factor-loaded NPs (50–150 nm), because growth factors complexed with heparin can promote a tighter interaction and more compact NP formation. NPs loaded with bFGF did not entirely coat the surface of PLGA microspheres, in contrast to the two-dimensional substrates in the geometrical structure of the microspheres (Fig. 2). However, growth factor-loaded NPs were shown to be immobilized on more than 50% of the PLGA microsphere surface, which would have been sufficient for binding-site recognition when the cells were seeded.

Determination of NP formation *via* visualization of FITC-labeled heparin and TRITC-conjugated PLL by CSLM is shown in Fig. 3A and 3B. In addition, merged images of microspheres and the completely overlapped image of bFGF-loaded NPs fluorescently labeled by several

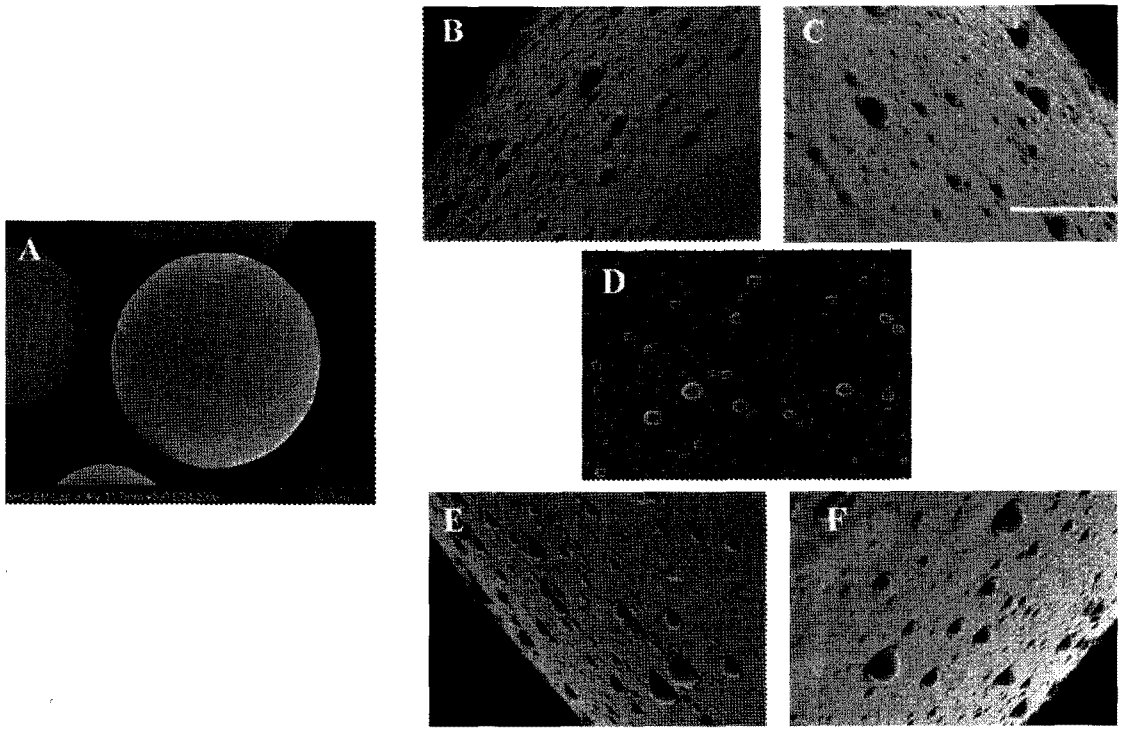

Fig. 2. SEM images of PLGA microspheres coated with heparin/PLL nanoparticles.
(**A**) Entire morphology of a PLGA microsphere; (**B**) upper left, (**C**) upper right, (**D**) lower right, (**E**) lower left, and (**F**) central regions of a microsphere.

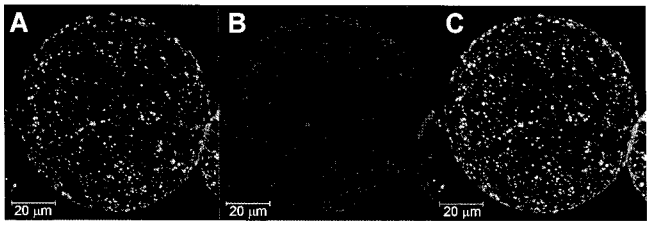

Fig. 3. Confocal scanning laser micrographs of heparin/PLL nanoparticles attached to PLGA microspheres.
A. FITC-conjugated heparin detected using green wavelength. **B**. TRITC-conjugated PLL detected using red wavelength. **C**. Merged images.

types of methods are shown in Fig. 3C. These results indicate that the negatively charged FITC-labeled heparin readily complexed with the positively charged bFGF, and then complexed again with TRITC-labeled PLL. In these images, the FITC fluorescence was very weak because the heparin complexed with bFGF in advance. From these results, it is clear that the positively charged PEI readily coated the negatively charged PLGA microspheres, and the heparinized NPs loaded with bFGF adhered to the PEI-coated surfaces of the microspheres.

PLGA microspheres coated with bFGF-loaded NPs exhibited a high efficacy of growth factor encapsulation and significant protein stability. Protein-loading efficiency was evaluated by bFGF-specific monoclonal antibody staining, which confirmed the presence of the protein (Fig. 4). The highest concentration of bFGF loaded onto NPs showed the greatest fluorescence. Since the negatively charged heparin entraps the positively charged bFGF of the polyplexed NPs on the surface of the PLGA microspheres, it is possible that heparin sulfate groups may interact with labile proteins, thereby reducing the availability of bFGF while protecting it from degradation. However, results of the monoclonal antibody assay confirm the availability of bFGF on the surface of the microspheres.

A cumulative release profile was obtained by monitoring the release of growth factor from PLGA microspheres (100 mg) *in vitro* in aqueous solution, using an initial concentration of 100 ng/ml bFGF (Fig. 5). The bFGF-loading efficiency of the sample calculated by ELISA was approximately 93% (data not shown). bFGF released from polyplex NPs immobilized on PLGA microspheres demonstrated a zero-order release profile during 4 weeks. This result suggests that growth factor released from such microspheres could help to increase neurite outgrowth of PC12 cells attached to the microspheres. To examine whether changes in secondary structure are reversible, growth factor released from polyplex NPs on PLGA microspheres was examined in a previous study. It was revealed that growth factor has the potential to maintain bioactivity following release from microspheres [15].

To investigate the effects of bFGF-loaded polyplex NPs, PC12 cells were seeded on NP-coated PLGA microsphere surfaces and incubated for 5 days (Fig. 6A–6C). Early in cultivation, PC12 cells were found to be attached directly to microsphere surfaces only, as shown in Fig. 6A. As cultivation continued, cell numbers increased and cells proliferated on the microspheres as shown in Fig. 6C. This result demonstrates that bFGF loaded on polyplexed NPs on PLGA microspheres has the ability to trigger not only differentiation but proliferation of PC12 cells attached to the microspheres.

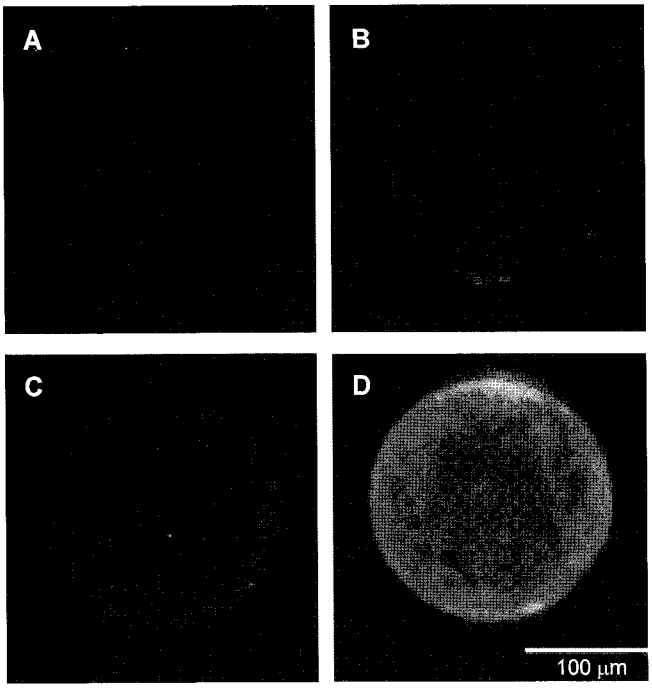

Fig. 4. Dose-dependent binding of bFGF-loaded nanoparticles on PLGA microspheres detected using bFGF-specific monoclonal antibody.
(**A**) 0, (**B**) 10, (**C**) 50, and (**D**) 100 ng/ml bFGF.

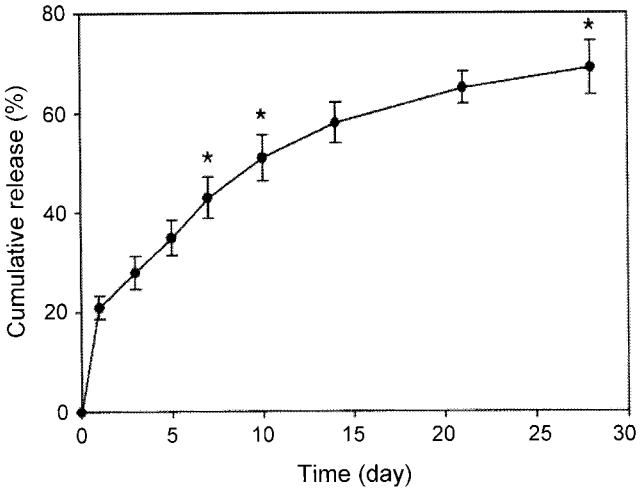

Fig. 5. Cumulative bFGF release profile from a bFGF-loaded NP-coated PLGA microsphere matrix, determined by ELISA.

Fig. 6. Fluorescent micrographs of PC12 cells adhered to PLGA microspheres and stained with PI.
Images were taken after (**A**) 1, (**B**) 3, and (**C**) 5 days of cultivation.

The effects of bFGF-loaded NP-coated PLGA microspheres on biological factors in PC12 cells were identified using the β-tubulin neuronal marker and observed by CSLM (Fig. 7). Other researchers have reported that the amount of the neuron-specific cytoskeletal protein β-tubulin was increased during growth factor-induced differentiation of PC12 cells [16]. Schmidt *et al.* [19] have suggested a number of potential mechanisms for matrix effects on PC12 neurite outgrowth using an electrically conducting polymer, some of which may apply here: participation of proteins involved in growth cone migration, such as cell membrane growth factor and adhesion receptors or cytoskeletal proteins; favorable changes in membrane or extracellular matrix protein conformation; direct nerve depolarization or hyperpolarization; increased protein synthesis; and generation of ionic and molecule gradients in the medium. Thus, when examining the maximum extent of bFGF-induced increase in neurite outgrowth, we compared the effect of culturing PC12 cells on bFGF-loaded NP-coated PLGA microspheres with that on microspheres alone and NP-coated microspheres to take into account all types of cell-matrix interactions. As shown in Fig. 7, neurite outgrowth in PC12 cells was stimulated significantly by bFGF immobilized on NP-coated microspheres, resulting in significant growth of neurite axons, whereas growth of neurite axons was not apparent in cells seeded on conventional microspheres alone. β-Tubulin (green fluorescence) was positively expressed in PC12 cells in neurite axons stimulated by bFGF (Fig. 7C). These results suggest that PC12 cells attached to PLGA microspheres coated with bFGF-loaded nanoparticles expressed functional acetylcholine and purinergic receptors while differentiating [12, 14] (β-tubulin-positive cells). During the extension of a neurite in a regenerating nerve,

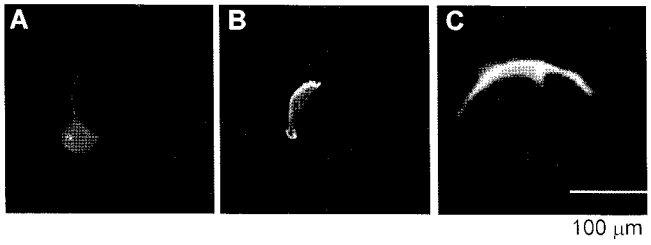

Fig. 7. Immunohistochemical assay of β-tubulin expression stimulated by (**A**) PLGA microspheres, (**B**) NP-coated microspheres, and (**C**) bFGF-loaded NP-coated microspheres.

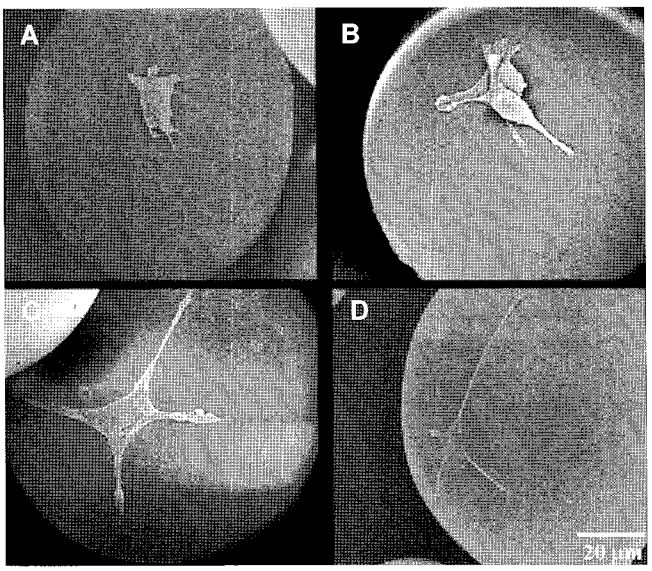

Fig. 8. SEM of morphology of PC12 cells adhered to bFGF-loaded NP-coated microspheres.
Images were taken (**A**) 1, (**B**) 2, (**C**) 3, and (**D**) 5 days after cell seeding.

the nerve growth cone will release growth factor *via* proteolysis, making it available to that neurite [18]. In addition, the bioactivity of the growth factor may be protected by its association with binding sites in the matrix material while it remains bound [18].

Fig. 8 shows SEM micrographs of neurite outgrowth in PC12 cells adhered to bFGF-loaded NP-coated microspheres over time. One day after seeding, PC12 cells were almost fully adhered to the microsphere surface and began to show formation of neurite outgrowth (panel A). Extended healthy neurites were observed on microsphere surfaces after 5 days of culture (panel D). These results indicate that the growth factors were successfully immobilized by heparin and that bFGF-loaded NPs are capable of sustaining primary nerve cells and providing the necessary support that is critical for regeneration. Neurite ourgrowth on growth factor-loaded NP-coated PLGA surfaces extended farther than that on NP-coated microspheres or PLGA microspheres only (data not shown). Even if cell adhesion on the NP-coated surface is superior to that on PLGA microspheres alone, significant differences in neurite outgrowth were not observed between them.

In summary, the use of bFGF-loaded NP-coated PLGA microspheres facilitates biological stimulation that is focused in the vicinity of adhered nerve cells, allowing for spatial control of stimulation. This technology should prove useful for injectable carriers for nerve regeneration.

Acknowledgments

This research was financially supported by the Ministry of Knowledge Economy (MKE) and the Korea Industrial

Technology Foundation (KOTEF) through the Human Resource Training Project for Strategic Technology and Fundamental R&D program for core Technology of Materials, Ministry of Konwledge Economy, Republic of Korea.

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