Tissue Engineering and Regenerative Medicine, Vol. 9, No. 2, pp 84-91 (2012)
DOI 10.1007/s13770-012-0034-4

| Original Article |

Evaluations of Nerve Cell Compatibility of Self Cross-Linking Chitosan-Poly(Ethylene Oxide) Hydrogel

Sumi Kim[1], Seongyeon Jo[1], Eunkyung Shin[1], Doyeon Kim[1,2], and Insup Noh[1]*

[1]*Department of Chemical and Macromolecular Engineering Seoul National University of Science and Technology 232 Gongnung-ro, Nowon-gu, Seoul, 139-743 Korea*

(Received: November 11th, 2011; Revision: December 20th, 2011; Accepted: December 23rd, 2011)

Abstract : Chitosan-poly(ethylene oxide) (chitosan-PEO) hydrogel was evaluated by the assays of live & dead, cell counting kit-8 (CCK-8), thiazoyl blue tetrazolium bromide (MTT) and neutral red for its applications in induction of adhesion, proliferation and differentiation of nerve cells by releasing nerve growth factors from the hydrogel. Fabrication of the chitosan-PEO hydrogel was obtained by mixing the precursor solutions of PEO with thiol groups and chitosan-acrylate, which was synthesized in advance by sequential chemical cross-linking of *para*-aminosalicyclic acid and 2-carboxyethyl acrylate to chitosan. After chemical characterizations of chitosan derivatives with FTIR and NMR, the chitosan-PEO hydrogels were evaluated by observing gel formation, and both biocompatibility and behaviors of PC-12 nerve cells. The nerve cells adhered on the hydrogel, and their adhesion and proliferation were affected by delivery of nerve growth factors (NGFs) from the hydrogels. Differentiation of PC-12 cells on the surface of the tissue culture flask was dependent upon the amount of NGFs sustain-released from the chitosan-PEO hydrogel as observed by the measurement of neurite length.

Key words: *chitosan, poly(ethylene oxide), nerve cells, differentiation, hydrogel*

1. Introduction

Chitosan, a linear polysaccharide composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is obtained commercially by deacetylation of chitin, which is a structural element in the exoskeleton of crustaceans such as crabs and shrimps. It demonstrated biocompatibility and biodegradability with minimal immune rejection[1, 2] and good mechanical properties[3] and hemostaticity as well as healing, antibacterial and antifungal properties.[4, 5] Based on these biocompatible properties, many types of chitosan biomaterials have been reported such as hydrogel,[6-10] film, porous scaffolds,[11,12] membrane,[13] fibers and nano- and micro-particles[14] depending on its applications in drug delivery,[15-17] tissue engineering and cell therapy.[18] Chitosan was also reported as scaffolds for nerve guide and angiogenesis[6,7] and for site-specific antibiotic and DNA delivery in the stomach.[19, 20] Adherence and survival of Schwann cells were demonstrated on a chitosan substratum *in vitro*, and good biocompatibility with regenerating sciatic nerves *in vivo*[21, 22] thus reinforcing the suitability of chitosan as a scaffold polymer for neural tissue engineering.[23] Furthermore chitosan has been employed as a hydrogel network for sealing and repairing damaged spinal cord nerve cells with PEO by R. Borgens *et al*, as scaffolds for neuronal growth in three dimensions to increase neuronal attachment by fabricating a mixture gel with agarose.[24] Chitosan was also employed as a nanoparticle for delivery of proteins and drugs.[25-27] Different ratios of chitosan and PEO in the fabrication of chitosan-PEO biomaterials were employed for designs of both its biological and drug delivery functions[6, 28] and their mechanical and surface properties.[29, 30]

Chitosan-PEO hydrogel was in this study fabricated by employing chitosan-acrylates and PEO-thiols as precursor solutions for cross linking of both precursor solutions through the Michael type addition reaction. Its properties were controlled by changing their concentrations and compositions. After verification of chemical grafting of acrylates to chitosan, the biological properties of the chitosan-PEO hydrogels were evaluated by employing PC-12 nerve cells. Adhesion, proliferation and differentiation of nerve cells were also evaluated by delivering different amount of nerve growth factors from the chitosan-PEO hydrogel.

*Tel: +82-2-970-6603; Fax: +82-2-977-8317
e-mail: insup@seoultech.ac.kr (Insup Noh)

2. Materials and Methods

2.1 Materials

Chitosan (85% deacetylated, MW:5-10 kDa) and polyethylene oxide (PEO) with *hexa*-thiols (MW : 10 kDa) were purchased from Kitto Life Co. (Seoul, Korea) and Sunbio (Seoul, Korea), respectively. While *para*-aminosalicylic acid (MW : 153 Da) and 2-carboxyethyl acrylate were purchased from Sigma-Aldrich (St. Louis, MO, USA), N-(3-diethylpropyl)-N-ethylcarbodiimide hydrochloride (EDC) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). RPMI1640 and penicillin-streptomycin were purchased from Lonza (Switzerland), and fetal bovine serum (FBS) was purchased from Gibco (Australia). Cell counting kit-8 (CCK-8) solution and live & dead viability/cytotoxicity kit for mammalian cells were purchased from Dojindo Laboratories (Japan) and Invitrogen (USA), respectively. All chemicals were employed as received.

2.2 Acrylation of Chitosan

Acrylation of chitosan was processed as follow. After dissolving chitosan (0.2 g) and PAS (0.14 g) in 20 mL distilled water and 20 mL methanol, respectively, 0.14 mL EDC was added into the mixture solution. PAS was grafted to chitosan at pH 4.75 for 24 hr after adding up EDC into the mixture solution, thus utilizing a final concentration of 1:1:1 (chitosan : EDC : PAS) in a molar ratio. Chitosan-PAS was lyophilized overnight after its precipitation in organic solvent. Chitosan-PAS was acrylated at pH 4.74 for 4 hrs by adding up 0.28 mL 2-carboxyethylacrylate (acrylate) and 0.41 mL EDC into 0.6% chitosan-PAS solution (w/v). Powder samples (chitosan-acrylate) were obtained by lyophilizing overnight after their precipitation in organic solvent.

2.3 Chemical Analysis

2.3.1 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

To observe acrylation of chitosan, ATR-FTIR spectra of chitosan, chitosan-PAS and chitosan-acrylate samples (0.01 g powder) were recorded at the wavelength of 650 to 4000 cm^{-1} with a spectrometer (Travel IR; Smiths, USA). While a diamond crystal refractive index was measured as 2.4 at 45^{o} incidence angle, the ATR depth of penetration was controlled as about 2 μm.

2.3.2 ^{1}H Nuclear Magnetic Resonance (^{1}H NMR)

^{1}H-NMR spectra were obtained by employing a mercury 400 MHz/CP-MAS spectrometer (Varian, Japan) to observe new chemical peaks in chitosan. Spectra of the chitosan, chitosan-PAS and chitosan-acrylate were measured with 1% D_2O solution (w/v).

2.4 Fabrication of Chitosan-PEO Hydrogel

Dry chitosan-acrylate powder (0.03 g) was dissolved in 300 μL RPMI-1640 medium in a polyethylene micro-centrifuge vial. In a separate vial, 0.03 g PEO-thiols was dissolved in 300 μL RPMI-1640 medium. The solutions of both the chitosan-acrylate and PEO-thiols were separately sterilized by filtering them through a 0.2 μL syringe filter (Corning: USA). Chitosan-PEO hydrogel was fabricated by mixing the above two precursor solutions.

2.5 *In Vitro* Evaluation of Chitosan-PEO Hydrogel

2.5.1 *In vitro* Cell Culture

In vitro cell culture was performed with PC-12 nerve cells (neural cell line) in RPMI-1640 medium supplemented with 10% fetal bovine serum. The medium contained 100 units/mL penicillin-streptomycin and gentamycin (2 μL /mL). The cells were cultured either on the surface of or in 200 μL chitosan-PEO hydrogel at densities of approximately either 1×10^{4} cells/surface or 1×10^{5} cells/encapsulation in a 24-well culture plate. Cellular behaviors on the hydrogels were quantitatively and qualitatively measured after seeding PC-12 cells at a density of 10,000 cells per sample by CCK-8 assay with a microplate reader (Tecan: Australia). CCK-8 solution (100 μL) was inserted into the 900 μL RPMI-1640 cell culture medium and then the cell culture plate was inserted in the 5% CO_2 incubator at 37^{o}C for 2 hr. 100 μL medium with CCK-8 solution was aliquoted into a 96 well plate, and then an optical density of the medium solution was measured at the wave length of 450 nm by the microplate reader.

2.5.2 Live & Dead Assay

In vitro biocompatibility of the solutions of chitosan (200 μL per sample), and the 10% chitosan-PEO hydrogel was evaluated with nerve cells (PC-12) at a density of 1×10^{5} cells/200 μL in a 24-well culture plate. Cell culture was performed for 6 hr in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin. Live & dead assay of the cells in the above samples was evaluated by fluorescence microscope. Teflon and Latex, 1 cm×1 cm, were employed as positive and negative controls, respectively.

2.5.3 Thiazolyl Blue Tetrazolium Bromide (MTT Assay)

PC-12 cells were *in vitro* cultured in a 96 well plate at a density of 1×10^{4} cells for 24 hr by employing the RPMI-1640

medium. Cell culture lasted for another 24 hr after addition of 200 μL hydrogels and Teflon and Latex. After adding up 20 μL MTT solution in the culture medium, cell culture lasted for another 4 hr. The culture medium with MTT solution was replaced with 100 μL dimethylsulfoxide. The optical density of the final solution was measured by the microplate reader at the wavelength of 570 nm.

2.5.4 Neutral Red Assay

Separate extract solutions of the hydrogel, Teflon and Latex were prepared after loading them in 1 mL culture medium for 72 hr. *In vitro* cell culture lasted for 24 hr after seeding nerve cells on the samples at a density of 1×10^4 cells/well. The prepared extract solution was added into and then the cell culture lasted for another 24 hr. After adding up 0.33% neutral red solution for 2 hr, and removal of the cell culture medium, fixation solution of neutral red assay was proceeded for 10 min with addition of 100 μL solubilization solution per well. Measurement of optical density was performed at the absorbance wavelength of 550 nm by the microplate reader by referencing that of 690 nm.

2.5.5 Differentiation of PC-12 Cell by NGF Release from Chitosan-PEO Hydrogel.

Proliferation and differentiation of PC-12 nerve cells were evaluated by sustained release of nerve growth factors from the chitosan-PEO hydrogel. PC-12 cells were culture for 24 hr after seeding them on the 10 cm polystyrene tissue culture flask at a density of 100,000 cells/dish. *In vitro* PC-12 cell cultures lasted for 9 days by changing the medium every 3 days with observation of development of neuritis with a light microscopy. Fabrication of NGFs-containing chitosan-PEO hydrogel was obtained in advance as follow. After mixing NGFs in the 10% solution of chitosan-acrylate in RPMI-1640 cell culture medium at the concentrations of either 200 or 800 ng/ml, chitosan-PEO hydrogel was formed by mixing it with 10% PEO solution. We measured the length of the neurite developed from the PC-12 cells during *in vitro* cell culture with light microscopy with image processor (UTHSCSA Image Tool 3.0; Samwoo Science Co., Seoul, Korea).

2.5.6 Immunocytochemistry

After fixing the *in vitro* cultured PC12 nerve cells in the chitosan-PEO hydrogel with paraformaldehyde for 10 min, the samples were washed with PBS. Monoclonal antibodies specific to neuron β-III tubulin (5 μg/mL, Abcam: England) were used to visualize neurite development. Fluorescein isothiocyanate (FITC)-conjugated secondary antibody was diluted into 1:1000 for 1h. After mounting the samples on the cover-glass, the nuclei of the cells were stained with 4,6-

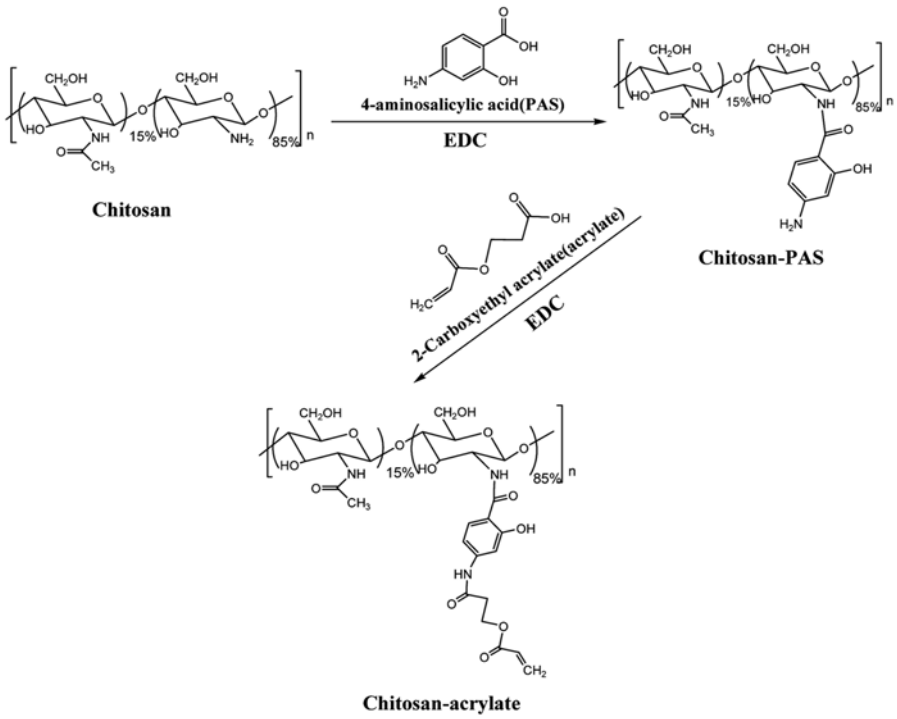

Figure 1. Schematics of synthesis of chitosan derivative grafted with *para*-aminoalicyclic acid (PAS) and carboxyethylacrylates (chitosan-acrylate).

diamidino-2-phenylindole (DAPI, Abcam) and then photographed using a fluorescence microscope (Leica DMLB, Germany).

2.6 Statistical Analysis

Data were reported as mean ± standard deviation. Statistical significance was assessed with one-way and multi-way ANOVA of the SPSS 12.0 program. The samples were considered as significantly different when $p < 0.05$.

3. Results

3.1 Synthesis of Chitosan-Acrylate

Chitosan-acrylate was synthesized by sequential grafting of PAS and carboxyethyl acrylate to chitosan through EDC chemistry, which is a reaction between the amine groups and 2-carboxylic acid (Fig 1). Grafting of PAS and carboxyethyl acrylate to chitosan was analyzed as follow with both FTIR and NMR.

3.2 Chemical Analysis

3.2.1 FT-IR Spectra of Chitosan Derivatives

FTIR peaks of chitosan, chitosan-PAS and chitosan-acrylate were compared as shown in Fig 2 to observe sequential grafting of PAS and 2-carboxyethyl acrylate to chitosan. Clear new chemical peaks were observed from the spectrum of the chitosan-acrylate. Typical FTIR peaks from chitosan were observed such as OH stretch vibration at 3260 cm^{-1}, N-H symmetrical vibration at 2400 cm^{-1}, carbonyl (C=O) stretch vibration at 1618 cm^{-1}, CH stretch vibration at 3007 cm^{-1} and 2880 cm^{-1}, C-N stretch vibration at 1558 cm^{-1} and CH_3 bend vibration at 1375 cm^{-1} (Fig 2A). Grafting of PAS and carboxyethyl acrylate was clearly observed from the new peaks at 3112 cm^{-1} for =C-H aromatic stretch vibration and 1368 cm^{-1} and at 1698 cm^{-1} for C=C aromatic vibration (Fig 2B and 2C).

3.2.2 NMR Spectra of Chitosan Derivatives

Chemical analyses of chitosan derivatives were performed with ^1H NMR by comparing the peaks of native chitosan with those of chitosan-PAS, chitosan-acrylate (Fig 3). The chitosan spectrum demonstrated its typical peaks at 1.78 ppm for CH_3, at 2.95 ppm for the second carbon peak, at 3.64 ppm for the third-sixth carbon peak, and at 4.45 ppm for the first carbon peak (Fig 3A). Grafting of PAS was clearly observed, as judged by the new peaks at 6.0 and 6.4 ppm on the chitosan-PAS spectrum (Fig 3B). While the peaks at 6.0 and 6.4 ppm were considered as the hydrogen atoms from the acrylate, that at 1.81 ppm was considered as the hydrogen atoms in the methyl

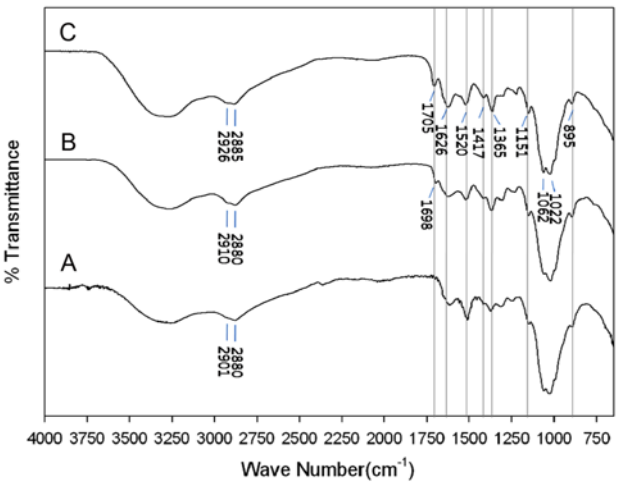

Figure 2. FT-IR spectra of (A) chitosan, (B) chitosan-PAS, and (C) chitosan- acrylate.

groups connected to the carbonyl group of the amide from the chitosan.[4, 9] Chitosan-acrylate spectra demonstrated new peaks, indicating successful grafting of PAS and 2-carboxyethyl acrylate such as -CH=CH- at the peak positions of 5.25 and 5.43 ppm, $-CH_2-CH=CH-$ at 2.06 ppm, $-OCO-CH_2-$ at 2.21 ppm, $=HC-CH_2-CH=$ at 2.71 ppm, CH_3 at 1.76 ppm, and $-CH_2-$ at 1.35 ppm (Fig 3C). Acrylation of chitosan was clearly observed from the peak at 5.43 ppm (Fig 3C). While the peaks at 5.25 and 5.47 ppm were considered as the indication from the acrylates of the hydrogel, the peak at 1.8 ppm was as that of methyl group in amide [refer to the chemical structures in Fig 1]. Degree of grafting of acrylate to chitosan was measured as 3.17% for chitosan-acrylate. It was estimated by calculating the ratio of the peak areas between acrylate and back bone at 2.71 ppm and 4.5~5.5 ppm, respectively.

3.3 Fabrication of Chitosan-PEO Hydrogel

Fabrication of chitosan-PEO hydrogel was obtained by mixing the precursor solutions of both chitosan-acrylate and PEO-thiols. The gel was spontaneously fabricated in several hr and demonstrated light yellow color. It was swollen over time and clear in water, and its shape remained stable over time.

3.4 *In Vitro* Cellular Behaviors of Chitosan Solutions and Hydrogel

In vitro cellular behaviors of chitosan-PEO hydrogel was evaluated by employing 10,000 nerve cells/200 μL and 10% hydrogel. Before evaluation of the hydrogel, biocompatibility chitosan-acrylate solutions at the concentrations of 1 and 5% was evaluated by live & dead assay of nerve cells for 24 hr (Fig

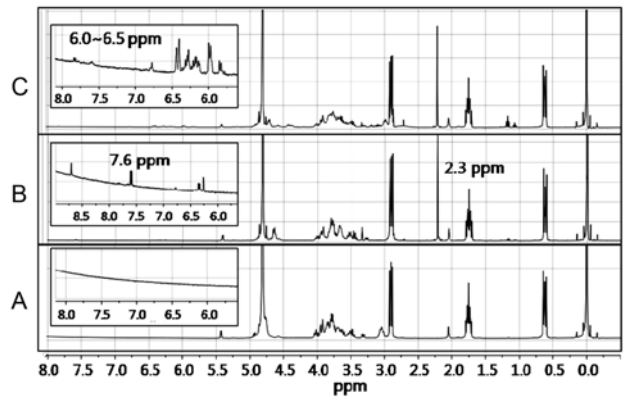

Figure 3. ^{1}H- NMR spectra of (A) chitosan, (B) chitosan-PAS, and (C) chitosan-acrylate.

4). While the cells in the 1% chitosan-acrylate solution were well viable at 24 hr (Fig 4A and 4D), but the 5% chitosan-acrylate solution induced severe cell damage (Fig 4B and 4E). Cell compatibility of the 10% chitosan-PEO hydrogel (50:50) was different when evaluated by seeding the nerve cells either inside or on the surface of the gel. Overall the cells on the surface of and inside the hydrogel were viable compared to those of the chitosan-acrylate solutions (Fig 4C and 4F).

3.5 *In Vitro* Cytotoxicity of Hydrogel

Biocompatibility of the chitosan-PEO hydrogels was evaluated to observe cell toxicity of specific cell organs by the assays of both MTT and neutral red, i.e. for their effects on possible damages of mitochondria and lysosome, respectively. Their biocompatibility was compared with those of Teflon and Latex as positive and negative controls, respectively.

MTT assay was to measure conversion of dehydrogenase in mitochondria into insoluble state. All the samples demonstrated significantly better cell viability than that of Latex, but less than that of positive control Teflon (Fig 5) ($p = 0.003$). The 10% hydrogel showed 83% cell viability over that of Teflon, while the Latex demonstrated 12% only.

The neutral red assay of the hydrogels demonstrated 60% viability of lysosomes by the vital dye, neutral red (basic red 5, toluylene red), compared to that of Teflon. While viable cells take up the neutral red by active transport and then incorporate it into lysosome, non-viable cells do not take up it. Latex induced very high extent of lysosomal damages, showing only 4% cell viability (Fig 5). This cell viability results indicated that less transport and accumulation of neutral red dye by lysosomes were induced by the effects of these gel extracts, but cell viability was improved by washing of the hydrogel with buffer solutions. In statistics, all the cell viability data were significantly different between samples ($p=0.002$).

3.6 Effects of NGFs Released from Chitosan-PEO Hydrogel on Nerve Cell Behaviors

Effects of NGF release on adhesion, proliferation and differentiation of nerve cells were observed at day 1, 3 and 7 on the chitosan-PEO hydrogels at the compositions of 25:75 and 50:50 by delivering 100 and 300 ng NGF/mL hydrogel in bolus. Cell adhesion and proliferation on the hydrogel were quantitatively measured with the values of the optical density by employing CCK-8. Cell adhesions on both 25:75 and 50:50 hydrogels were negligibly small and very small degrees of cell proliferation were observed over 7 days when 100 ng NGF/mL was released from the hydrogel in the culture medium (Fig 6). However when 300 ng NGF was delivered in bolus, the

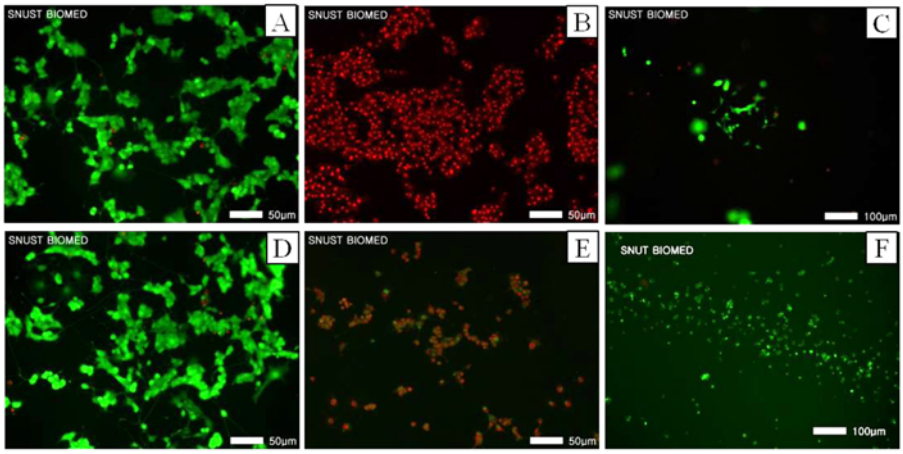

Figure 4. Live & dead assay of PC-12 cells for 24 hrs in the solutions of chitosan and chitosan-acrylate 1% (A and D) and 5% (B and E), and inside the 10% chitosan-PEO hydrogel (50:50) (C and F).

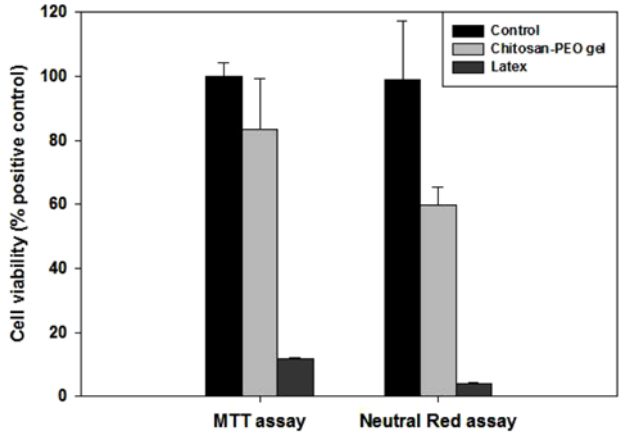

Figure 5. Cytotoxicity assay of chitosan-PEO gel (50:50) with PC-12 cells after cell culture for 48 hrs including 24 hrs cell culture in extracts.

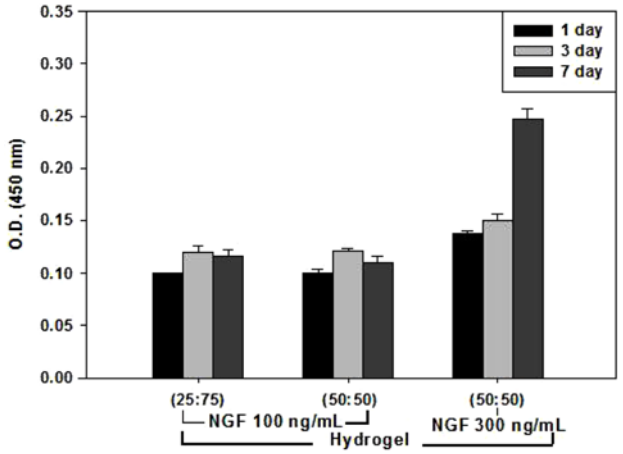

Figure 6. Proliferation of PC-12 cells on the chitosan-PEO (50:50) hydrogel after delivering nerve growth factors (NGF) in bolus.

hydrogel induced more cell adhesion even at day 1. Furthermore, cell proliferation was observed on the surface of the hydrogel over 3 and 7 days from 0.14 and 0.25 in their optical density values.

Morphological changes and differentiation of nerve cells *in vitro* cultured on the polystyrene culture dish were affected by delivering both 200 and 800 ng NGFs from the 200 μL chitosan-PEO hydrogel (50:50) for either 3 (Fig 7A, 7B and 7C) or 9 days (Fig 7D, 7E and 7F). Immunochemical staining demonstrated differentiation of nerve cells by observing nuclear stains in blue through β-III tubulin antibody and neurite differentiation in green by fluorescent microscopy (Fig 7G, 7H and 7I). While the control cells with no NGF delivered from the chitosan-PEO hydrogel showed round cell shapes (Fig 7G), indicating no nerve cell differentiation (Fig 7A and 7D), the cells with NGF delivered from the chitosan-PEO hydrogel showed different degrees of nerve cell differentiations depending on the cell culture times and the amount of NGFs. Very small amount of differentiation of nerve cells was observed over 9 days when delivered 200 ng NGFs/mL gel (Fig B, 7E and 7H). However when we delivered 800 ng NGF/mL gel, dramatic differentiations of nerve cells were observed over the initially seed cells (Fig 7C and 7F) as observed nerve cell nuclei in blue and differentiated β-III tubulin, (Fig 7I), respectively. Next we measured the length of the neurite developed from the PC-12 cells during *in vitro* cell culture (Fig 8). When we did not delivered NGF, negligible amount of neurite development was observed, i.e. 8.6 μm for 9 days. Dramatic development of neurite from the PC-12 cells was observed when we sustain-delivered NGF from the hydrogel over 9 days. While delivery of 200 ng NGF from the chitosan-PEO hydrogel induced *in vitro* development of Neurite extension to 94.1 and 104 μm for 3 and 9 days, that of 800 ng NGF induced 83.1 and 159.7 μm, respectively. The effects of sustain-delivery of NGF from the hydrogel on the neurite development were significantly different at day 9 ($p = 0.03$ between 0 and 200 ng NGF; and 0.01 between 200 and 800 ng NGF), even though there was no difference in statistic at day 3 ($p = 0.97$)

4. Discussion

The chitosan-PEO hydrogel was fabricated by mixing of the precursor solutions of both chitosan-acrylate and PEO-thiol via self-crosslinking mechanism of Michael type addition reaction. Chitosan-acrylate was synthesized by sequential grafting of PAS and carboxylethylacrylate, with expectations of antibiotic properties by PAS. Furthermore PAS was expected to give some mechanical properties to the hydrogel because of its benzene ring structure. While 1% chitosan-acrylate solution did not induce cell damages during cell encapsulation, 5% one induced cell death, indicating cell damages by the density of chitosan-acrylate solutions. On the contrary to these results, the chitosan-PEO hydrogel induced negligible cell damages both inside and on the surface of even 10% hydrogel. Nerve cell compatibility of the chitosan-PEO hydrogel was evaluated with assays of MTT and neutral red, by employing its extracts. The extracts from the hydrogel seemed to induce some damages to cell organs such as mitochondria and DNA, but washing of the hydrogel in advance with water led to better nerve cell viability, indicating that thorough removal of contaminants of the

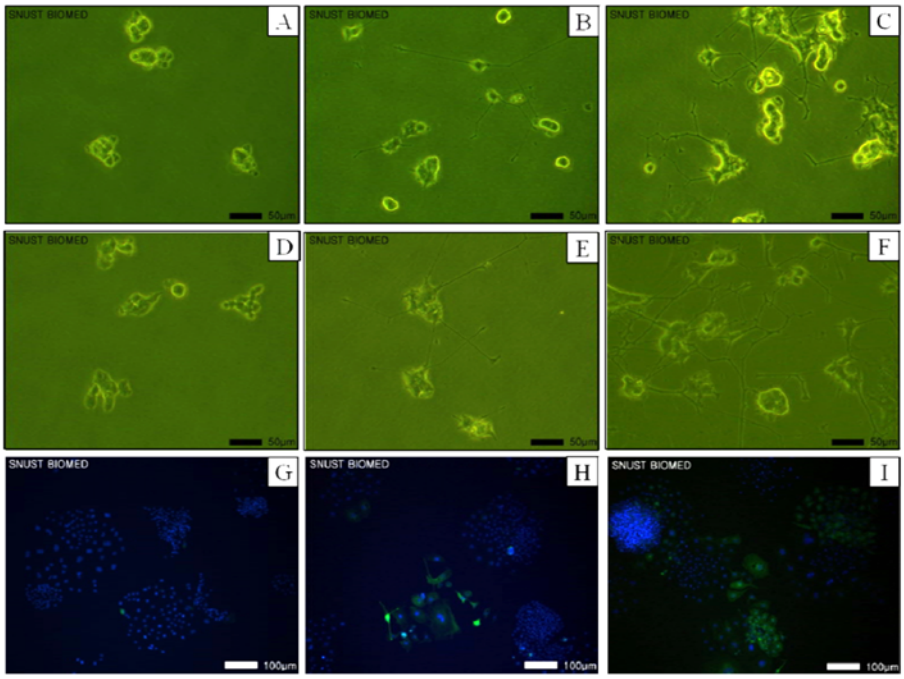

Figure 7. Morphology of PC12 cells *in vitro* cultured on the polystyrene culture dish affected by releasing NGFs for either 3 (A, B and C) or 9 days (D, E and F) and β-III tubulin staining (G, H and I; blue = nuclei, green = β-III tubulin); controls (A, D and G). NGF was sustainly released from the 200 μL chitosan-PEO (50:50) gel initial loading of either 200 (B, E and H) or 800 ng NGF/mL gel (C, F and I).

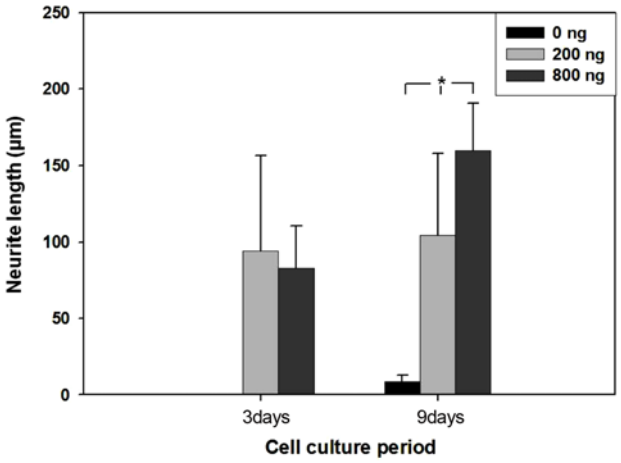

Figure 8. Dependence of the amount of NGFs sustain-released from the chitosan-PEO hydrogel on the length of *in vitro* developed neurites of the PC-12 cells for either 3 or 9 days.

chitosan-PEO hydrogel seemed to be of importance in obtaining biocompatible medical devices. Proliferation of nerve cells and differentiation of neuritis were affected by delivery of NGFs in mode and amount from the hydrogel. Even though both 300 ng NGFs in bolus and 200 ng NGFs per 1 mL chitosan-PEO hydrogel were not enough to induce proliferation and differentiation of nerve cells regardless of the compositions of chitosan and PEO (25:75 and 50:50 in this study) in the hydrogel, 800 ng NGFs per 1 mL chitosan-PEO hydrogel induced noticeable amount of neuritis differentiation, indicating that the cell compatible chitosan-PEO hydrogel could be employed for delivery of NGFs, with no damage to the biological properties of nerve cells and possibly growth factors inside and nearby the chitosan-PEO hydrogel.

Acknowledgements: This research was supported by the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2011-0001804).

References

1. H Onishi, Y Machida, Biodegradation and distribution of water soluble chitosan in mice, *Biomaterials*, **20**, 2 (1999).
2. Q Li, ET Dunn, EW Grandmaison, *et al*., Applications and properties of chitosan, *J Bioact Compat Polym*, **7**, 4 (1992).
3. F Yang, X Li, M Cheng, *et al*., Performance modification of chitosan membranes induced by gamma irradiation, *J Biomater Appl*, **16**, 3 (2002).
4. MNV Ravi Kumar, A review of chitin and chitosan applications, *React Funct Polym*, **46**, 1 (2000).
5. C Shi, Y Zhu, X Ran, *et al*., Therapeutic potential of chitosan

and its derivatives in regenerative medicine, *J Surg Res*, **133**, 2 (2006).
6. R Ahmadi, AJ Burns, Chitosan-based hydrogels do not induce angiogenesis, *Tissue Eng Regen Med*, **4**, 4 (2010).
7. M Patel, L Mao, B Wu, *et al*., GDNF–chitosan blended nerve guides: a functional study, *Tissue Eng Regen Med*, **1**, 5 (2007).
8. JL Drury, DJ Mooney, Hydrogels for tissue engineering: scaffold design variables and applications, *Biomaterials*, **24**, 24 (2003).
9. MS Kim, YJ Choi, I Noh, *et al*., Synthesis and characterization of in situ chitosan-based hydrogel via grafting of carboxyethyl acrylate, *J Biomed Mater Res A*, **83A**, 3 (2007).
10. D Kim, S Kim, S Jo, *et al*., Effect of cross-linking spacers on biocompatibility of chitosan-spacer-poly(ethylene oxide) hydrogel, *Macromol Res*, **19**, 6 (2011).
11. YC Kuo, YR Hsu, Tissue-engineered polyethylene oxide/ chitosan scaffolds as potential substitutes for articular cartilage, *J Biomed Mater Res A*, **91A**, 1 (2009).
12. MS Bae, DW Kim, SE Kim, *et al*., BMP-2 immobilized gelatin-β-chitosan scaffold for bone regeneration, *Tissue Eng Regen Med*, **6** (2009).
13. H Qianru, Z Tianyi, Y Yumin, *et al*., In vitro biocompatibility of chitosan-based materials to primary culture of hipocampal neurons, *J Mater Sci Mater Med*, **20** (2009).
14. Y Cho, R Shi, Chitosan nanoparticle-based neuronal membrane sealing and neuroprotection following acroleininduced cell injury, *J Biol Eng*, **4**, 1 (2010).
15. TT Minh, WY Baek, Controlled formulation for epinephrine delivery from hydrogel microspheres, *Tissue Eng Regen Med*, **6**, 1-3 (2009).
16. E Patois, S Osorio-da Cruz, JC Tille, *et al*., Novel thermosensitive chitosan hydrogels: In vivo evaluation, *J Biomed Mater Res A*, **91A**, 2 (2009).
17. Y Hong, Z Mao, H Wang, *et al*., Covalently crosslinked chitosan hydrogel formed at neutral pH and body temperature, *J Biomed Mater Res A*, **79A**, 4 (2006).
18. T Drewa, J Adamowicz, J Lysik, *et al*., Chitosan scaffold enhances nerve regeneration within the in vitro reconstructed bladder wall: an animal study, *Urol Int*, **81**, 3 (2008).
19. VR Patel, MM Amiji, Preparation and characterization of freeze-dried chitosan-poly(ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach, *Pharm Res*, **13**, 4 (1996).
20. IK Park, TH Kim, YH Park, *et al*., Galactosylated chitosan-graft-poly(ethylene glycol) as hepatocyte-targeting DNA carrier, *J Control Release*, **76**, 3 (2001).
21. A Wang, Q Ao, Y Wei, *et al*., Physical properties and biocompatibility of a porous chitosan-based fiber-reinforced conduit for nerve regeneration, *Biotechnol Lett*, **29**, 11 (2007).
22. G Wang, G Lu, Q Ao, *et al*., Preparation of cross-linked carboxymethyl chitosan for repairing sciatic nerve injury in rats, *Biotechnol Lett*, **32**, 1 (2010).
23. Q Ao, CK Fung, AYP Tsui, *et al*., The regeneration of transected sciatic nerves of adult rats using chitosan nerve conduits seeded with bone marrow stromal cell-derived Schwann cells, *Biomaterials*, **32**, 3 (2011).
24. Z Cao, RJ Gilbert, W He, Simple agarose-chitosan gel composite system for enhanced neuronal growth in three dimensions, *Biomacromolecules*, **10**, 10 (2009).
25. P Calvo, C Remuñán-López, JL Vila-Jato, *et al*., Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers, *J Appl Polym Sci*, **63**, 1 (1998).
26. H Jung, MK Jang, JW Nah, Synthesis and characterization of thermosensitive nanoparticles based on PNIPAAm core and chitosan shell structure, *Macromol Res*, **17**, 4 (2009).
27. BS Kim, TY Yeo, YH Yun, *et al*., Facile preparation of biodegradable glycol chitosan hydrogels using divinyladipate as a crosslinker, *Macromol Res*, **17**, 10 (2009).
28. CG Di, Y Zambito, S Burgalassi, *et al*., Effect of chitosan on in vitro release and ocular delivery of ofloxacin from erodible inserts based on poly(ethylene oxide), *Int J Pharm*, **248**, 1-2 (2002).
29. VL Alexeev, EA Kelberg, GA Evmenenko, *et al*., Improvement of the mechanical properties of chitosan films by the addition of poly(ethylene oxide), *J Polym Eng Sci*, **40**, 5 (2000).
30. MM Amiji, Synthesis of anionic poly(ethylene glycol) derivative for chitosan surface modification in blood-contacting applications, *Carbohydr Polym*, **32**, 3-4 (1997).