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Ionically cross-linked chitosan microspheres for controlled release of bioactive nerve growth factor

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ABSTRACT

Controlled release of neurotrophic factors to target tissue *via* microsphere-based delivery systems is critical for the treatment strategies of diverse neurodegenerative disorders. The present study aims to investigate the feasibility of the controlled release of bioactive nerve growth factor (NGF) with ionically cross-linked chitosan microspheres (NGF–CMSs). The microspheres were prepared by the emulsion-ionic cross-linking method with sodium tripolyphosphate (STPP) as an ionic cross-linking agent. The size and distribution of the microspheres, SEM images, Fourier transform infra red spectroscopy (FT-IR), encapsulation efficiency, *in vitro* release tests and bioactivity assay were subsequently evaluated. We found that the microspheres had relatively rough surfaces with mean sizes between 20 and 31 μm. FT-IR results provided evidence of ionic interaction between amino groups and phosphoric groups of chitosan and STPP. The NGF encapsulation efficiency ranged from 63% to 88% depending on the concentration of STPP. The *in vitro* release profiles of NGF from NGF–CMSs were influenced by the concentration of STPP. NGF–CMSs which were cross-linked with higher concentration of STPP showed slower but sustained release of NGF. In addition, the released NGF from NGF–CMSs was capable of maintaining the viability of PC12 cells, as well as promoting their differentiation. Taken together, our findings suggest that NGF–CMSs are capable of releasing bioactive NGF over 7 days, thus having potential application in nerve injury repair.

1. Introduction

Peripheral nerves possess the capacity for self-regeneration after injury. This process is dependent upon a lot of factors including permissive environment and the activation of the intrinsic growth capacity of neurons. Neurotrophic factors are known to play a vital role in controlling the neuronal survival and axonal regeneration at the nerve injury site (Fu and Gordon, 1997; Markus et al., 2002). Nerve growth factor (NGF), as an important member of neurotrophin family, not only promotes the survival and neurite outgrowth of sensory neurons both *in vitro* and *in vivo*, but also enhances peripheral nerve regeneration, as shown in many previous studies (Rich et al., 1989; Derby et al., 1993). However, the application of NGF in promoting nerve regeneration was limited by its short biological half-life and its vulnerability to structural disruption or modification, leading to loss of bioactivity. Therefore, protein drug delivery systems are needed not only to improve the biological utilization of NGF by sustained release of bioactive NGF to target site, but also to protect its bioactivity from degradation by direct exposure to harsh environments, *e.g.*, light, oxygen, chemicals, *etc.* In recent years, microspheres-based drug delivery systems, fabricated by biodegradable synthetic polymers such as poly(lactide-co-glycolic acid) (PLGA), have been widely reported in previous studies (Choi et al., 2002; Han et al., 2010). However, these synthetic materials have several inherent flaws, such as acidic degradation products, retarded clearance rate, and limited biological function (Freiberg and Zhu, 2004).

Natural polymers present an alternative choice to synthetic polymers for protein drugs delivery, including chitosan (Sinha et al., 2004), alginate (Jay and Saltzman, 2009) and hyaluronic acid (Zhao, 2006). Chitosan [poly (β-(1 → 4)-2-amino-2-deoxy-D-glucose)] is a natural linear polysaccharide obtained from alkaline N-deacetylation of chitin, a naturally abundant biocompatible polymer (Lee et al., 1997; Ravi Kumar, 2001). Owing to its favorable properties, such as good biocompatibility, biodegradability, low toxicity, and antimicrobial activity, chitosan has been developed for medical and biomedical applications including drug delivery and tissue engineering applications (MacLaughlin et al., 1998; He et al., 1999). Recent studies have shown that chitosan is a potential candidate biomaterial for nerve tissue engineering (Lu et al., 2007; Li et al., 2009; Huang et al., 2010a,b). In addition,

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chitooligosaccharides (COSs), the biodegradation products of chitosan, have been demonstrated *in vitro* to promote neuronal differentiation and neurite outgrowth (Yang et al., 2009). All these properties make chitosan an ideal substance for encapsulating NGF and for its sustained release in nerve injury repair. However, very few studies have reported on the encapsulation of NGF into chitosan microspheres prepared by emulsion-ionic cross-linking method, which hold great potential for improving nerve regeneration.

The present study was designed to prepare NGF–CMSs by emulsion-ionic cross-linking method. The bioactive molecules were incorporated into chitosan microspheres, which were then ionically cross-linked in the presence of STPP. The particle size of NGF–CMSs was analyzed by particle size analyzer; its surface characteristics were examined by SEM; the physicochemical interaction was evaluated by Fourier transform infra red spectroscopy (FT-IR); the kinetic release characteristics of NGF–CMSs were examined by an ELISA method. Finally, the bioactivity of the released NGF was investigated by PC12 cell bioassay and MTT assay.

2. Materials and methods

2.1. Materials

Chitosan (Brookfield viscosity >200,000 cps, degree of deacetylation ≥75%) and STPP were obtained from Sigma–Aldrich Co. Recombinant rat NGF and NGF enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich Co. (St. Louis, USA). Span 80 and other chemicals were all analytical grades and were used as received.

2.2. Preparation of NGF–CMSs

Chitosan microspheres were essentially fabricated by an emulsion-ionic cross-linking method described previously (Niu et al., 2009a,b). In brief, chitosan solution (2.0% (w/v)) was prepared by agitating chitosan in 10 ml of 2.0% (v/v) aqueous acetic acid solution at room temperature overnight. 1 ml of phosphate-buffered saline (PBS, pH 7.4) containing 6 μg of NGF and 0.15 mg of BSA was added to the chitosan solution, which was used as a water phase. Liquid paraffin (150 ml) containing 2.0% (v/v) surfactant span 80 was used as an oil phase (4 °C). The water phase was then dropped into the oil phase and agitated mechanically for 1 h at 4 °C to form water/oil (W/O) emulsion. Thereafter, 20 ml of STPP solution (1%, 3%, 5%, and 10% (w/v)) which was used as an ionic cross-linking agent was slowly added in droplets to the W/O emulsion to solidify the chitosan droplets for 1 h with mechanical stirring. The microspheres were then obtained by washing with petroleum ether and isopropyl alcohol repeatedly, prior to lyophilization (Alpha 2-4, Chaist, Germany). The dried microspheres were stored at 4 °C.

2.3. Surface characteristics and size distribution of the microspheres

The appearance morphology of the microspheres was examined by scanning electron microscopy (SEM; S-3400N; HITACHI, Tokyo, Japan). Freeze dried microspheres were mounted onto metal stubs with double-sided adhesive tape and then coated with gold in a vacuum. The particle mean size and size distribution of the microspheres were determined by a laser particle size analyzer (Mastersizer2000, UK). The dried microspheres were dispersed into deionized water and agitated on a shaker table at 300 rpm for more than 5 min. The obtained solution was used to determine mean size and size distribution.

2.4. Determination of NGF content in the microspheres

The content of NGF in NGF–CMSs was determined as follows: 30 mg of NGF–CMSs were dissolved in 10 ml of 0.1 N acetic acid and stirred in a vortex for 20 min at room temperature. The mixture was centrifuged at 1000 rpm for 10 min and filtered with a syringe filter (0.45 μm in pore size). The amount of NGF loaded in chitosan microspheres was assayed by NGF ELISA kit following the instructions of the manufacturer. All samples were analyzed in triplicate and the results were expressed as mean ± SD. Encapsulation efficiency (EE) was calculated as follows:

$$\mathrm{EE} = \frac{\text{actual NGF content}}{\text{theoretical NGF content}} \times 100\%$$

2.5. Fourier transform-infra red spectroscopy (FT-IR)

The FT-IR spectrophotometer (FTIR-8400S, SHIMADZU®, Japan) was used to record FT-IR spectra. Chitosan, unloaded chitosan microspheres samples and anhydrous potassium bromide (KBr) were used to form a thin pellet for analysis.

2.6. In vitro protein release study

30 mg of NGF–CMSs were dispersed in 6 ml of phosphate-buffered saline (PBS, pH 7.4). The samples were placed in a vibrating water bath at 37 °C and stirred at 100 rpm for up to 7 days. At the predetermined time points, the solution was centrifuged at 13,500 rpm. 200 μl of the release medium were withdrawn and frozen to −20 °C until analysis by ELISA with the commercially available kit. 200 μl of fresh medium were again added to the samples. The *in vitro* release experiments were performed in triplicate for each of the samples and the data were shown as mean ± SD.

2.7. Bioactivity of NGF released in vitro from NGF–CMSs

To confirm the bioactivity of the released NGF from NGF–CMSs, pheochromocytoma (PC12) cells were used as an assay system *in vitro*. PC12 cells differentiate into a neuronal phenotype by extending neurites in response to bioactive NGF (Hall et al., 1988). PC12 cells were cultured in T-75 flask at 1.0×10^5 cells/ml in 10 ml culture medium consisting of RPMI1640 (Gibco, USA), 10% horse serum (Gibco, USA), 5% fetal bovine serum (Gibco, USA) and 1% Penicillin/Streptomycin (Gibco, USA). The cells were seeded at a density of 1.0×10^4 cells/well in 1 ml culture medium on 24-well culture plate (Nunc, Roskilde, Denmark). At different times (1, 3, 5, and 7 days), a volume of 300 μl of the NGF supernatant released from NGF–CMSs was added to each well. Cultures were maintained at 37 °C in 5% CO_2 atmosphere for 72 h. The percentage of PC12 cells with positive response was determined under an optical microscope. At least 100 cells were counted in each well. PC12 cells with one or more neuritis longer than one cell body length were regarded as cells with positive response to bioactive NGF (Sakiyama et al., 1999). The positive control media was a NGF-supplemented medium (10 ng/ml) and the negative control media was the released media without NGF.

2.8. MTT assay

For viability testing, MTT (3-{4,5-dimethylthiazole-2-yl}-2,5-diphenyl tetrazolium bromide) chromometry assays were carried out (Kim et al., 2000; Hung et al., 2006; Kang et al., 2008). PC12 cells were plated in 96-well plates (Nunc, Roskilde, Denmark) at a density of 1×10^4 cells per well containing 200 μl of RPMI1640 (supplemented with 10% horse serum, 5% fetal bovine serum and 1% Penicillin/Streptomycin) in the absence (control group) or presence

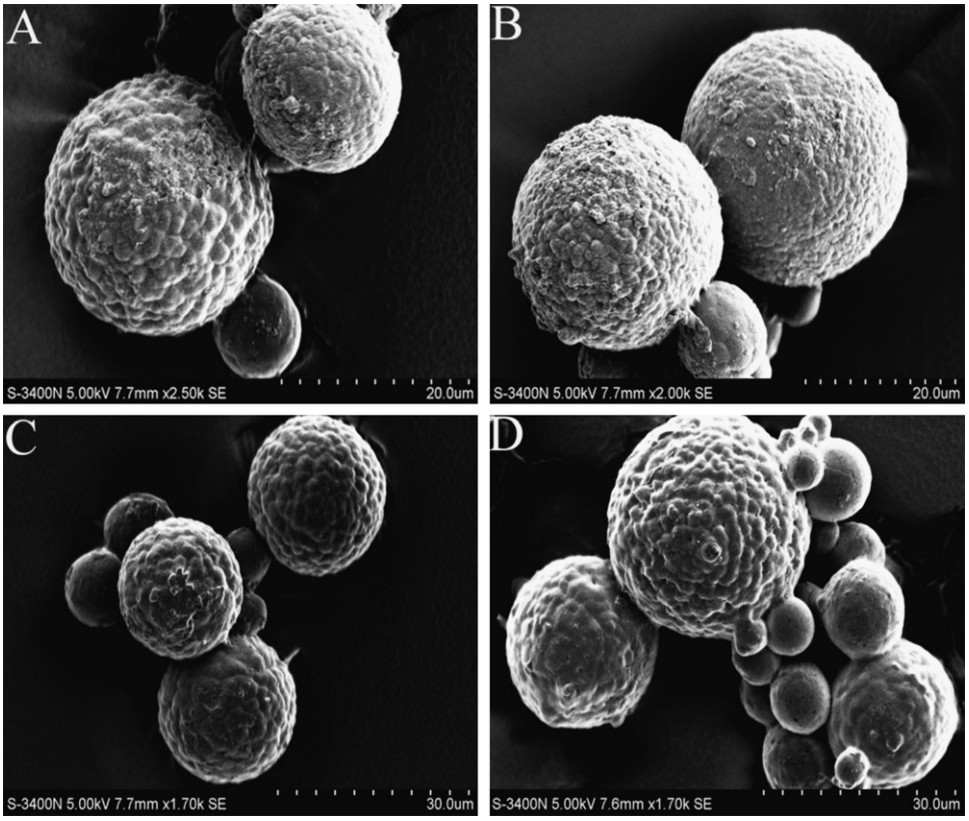

Fig. 1. Scanning electron micrographs of NGF-loaded chitosan microspheres, which were cross-linked 1% STPP (A), 3% STPP (B), 5% STPP (C), and 10% STPP (D).

of 10 ng/ml NGF (NGF group), 20 mg/ml NGF–CMSs (NGF–CMSs group, contains 10 ng/ml NGF) at 37 °C in humidified air containing 5% CO_2 for 1, 3, 5, and 7 days. After cultured period, 20 μl of MTT solution was added per well and the plates were incubated for 2 h at 37 °C on a rotating platform (250 rmp). After incubation, the media were removed and formazan crystals were dissolved into 150 μl DMSO. The plates were then read on a Microplate reader (Titertek, Helsinki, Finland) at 570 nm wavelength.

2.9. Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) with the SPSS13.0 software package (SPSS, Chicago, IL). Values with $p < 0.05$ were considered significant.

3. Results

3.1. Characteristics of NGF–CMSs

NGF–CMSs were prepared by the ionic interaction between a positively charged amino group of chitosan and a carrying five negatively charged STPP. NGF–CMSs ionically cross-linked with 1% STPP (Fig. 1A), 3% STPP (Fig. 1B), 5% STPP (Fig. 1C), or 10% STPP (Fig. 1D) showed relative rough surface morphology and spherical shape without hollows or deformations. The microspheres surfaces were not obviously different as the cross-linking degree increased.

The mean size and size distribution of NGF–CMSs were measured by Mastersizer particle size analyzers. The diameter distribution of the microspheres cross-linked with 3% STPP varied from 3 to 60 μm (Fig. 2). The mean size of NGF–CMSs cross-linked with different concentration of STPP was in the range of 20.5–31.2 μm (Table 1), which was strongly influenced by STPP

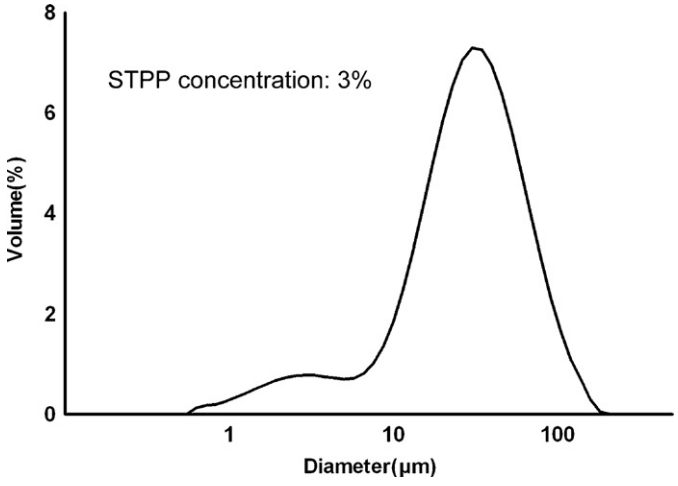

Fig. 2. Size distribution of NGF loaded chitosan microspheres cross-linked with 3% STPP.

Table 1
Effect of STPP concentrations on encapsulation efficiency, NGF loaded and mean size of NGF–CMSs.

STPP (%)	EE (%)	NGF loaded (ng NGF/mg CMSs)	Mean size (μm)
1	87.9 ± 2.2	12.52 ± 0.17	20.5 ± 1.2
3	80.1 ± 2.4[a]	6.15 ± 0.18[a]	22.8 ± 1.7[a]
5	71.2 ± 1.7[a,b]	3.54 ± 0.13[a,b]	26.7 ± 2.1[a,b]
10	62.8 ± 3.1[a,b,c]	1.78 ± 0.23[a,b,c]	31.2 ± 2.8[a,b,c]

Data are expressed as mean ± SD; EE, encapsulation efficiency.
[a] $p < 0.05$ when compared with 1% STPP.
[b] $p < 0.05$ when compared with 3% STPP.
[c] $p < 0.05$ when compared with 5% STPP.

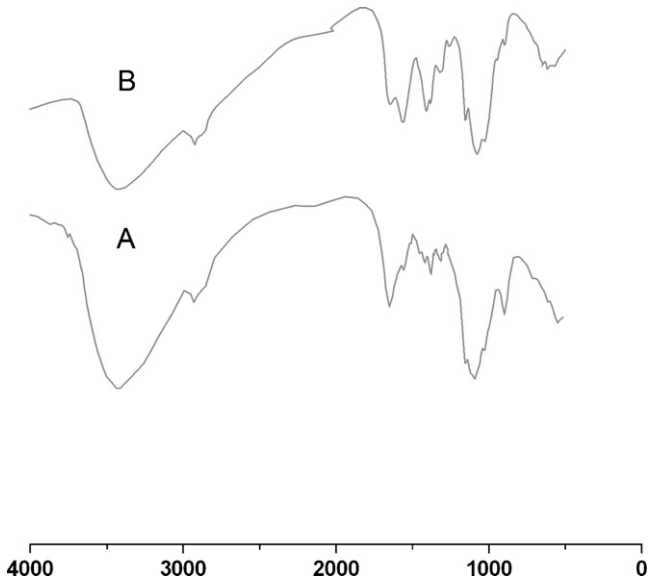

Fig. 3. FT-IR spectra of chitosan (A) and unloaded chitosan microspheres (B).

concentration. A significant increase was observed in the mean particle size of the microspheres while the concentration of STPP increased ($p < 0.05$). When the concentration of STPP was increased from 1% to 10%, the mean size of the microspheres increased from 20.5 ± 1.2 μm to 31.2 ± 2.8 μm.

The FT-IR spectra of chitosan and unloaded chitosan microspheres were shown in Fig. 3. In the FT-IR spectrum of chitosan (Fig. 3A), a broad absorption band at 3430 cm^{-1} corresponded to the stretching vibration of $-NH_2$ and –OH groups. The peaks at 2930 and 2848 cm^{-1} were typical of C–H stretch vibration, while the peaks at 1640, 1563 and 1313 cm^{-1} corresponded to amides I, II and III, respectively. The sharp peaks at 1426 and 1380 cm^{-1} attributed to the CH_3 symmetrical deformation mode and 1155 and 1078 cm^{-1} were indicative of C–O stretching vibrations [ν(C–O–C)]. The absorption band at 896 cm^{-1} was characteristic of saccharide structure of chitosan (Yuan et al., 2010).

From unloaded chitosan microspheres spectrum (Fig. 3B), it was found that the peak of 3430 cm^{-1} became wider, which implied that hydrogen bonding was significantly enhanced. The peak of $-NH_2$ bending vibration shifted from 1640 cm^{-1} to 1560 cm^{-1} and a new peak 1660 cm^{-1} appeared. This result was similar to the one reported by Knaul et al. (1999). It could be attributed to electrostatic interaction between phosphoric groups and ammonium ions.

In order to investigate the effect of STPP concentration on the encapsulation efficiency of NGF–CMSs, different concentrations of STPP were used to prepare NGF–CMSs. The amount of recombinant rat NGF encapsulated was determined by ELISA. As shown in Table 1, when STPP increased from 1% to 5%, the encapsulation efficiency of NGF decreased from 87.9 ± 2.2% to 71.2 ± 1.7%. When STPP concentration increased up to 10%, the encapsulation efficiency further decreased to 62.8 ± 3.1%. The amount of NGF loaded in the microspheres was between 1.78 ± 0.23% and 12.52 ± 0.17%. A significant decrease was observed in encapsulation efficiency and loading amount of NGF while the concentration of STPP increased from 1% to 10% ($p < 0.05$, Table 1).

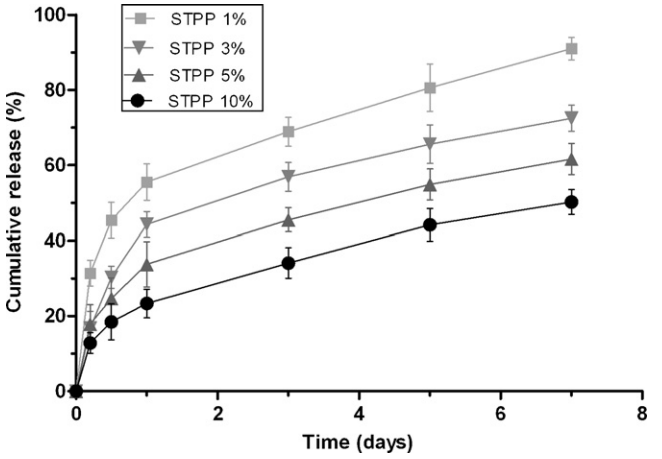

Fig. 4. *In vitro* release profiles of NGF–CMSs cross-linked with different STPP concentrations in PBS solution (pH 7.4). Error bars represent standard deviation.

3.2. In vitro protein release studies

The release kinetics of NGF from NGF–CMSs with ionic cross-linking with different STPP concentration was shown in Fig. 4. It revealed that the release profiles of NGF from the microspheres decreased when the cross-linking agent concentration increased. There were three stages for the release of NGF from the microspheres (Table 2). In the initial burst stage (0–12 h), the release rate was rapid, which was followed by the second (12 h–5 days) and third stage (5–7 days) with slow release. The amount of NGF released at the initial burst stage (0–12 h) decreased as the concentration of STPP increased. As shown in Table 2, during the first 12 h, the microspheres cross-linked with 1% STPP exhibited an initial release of 45.5% as compared to 30.3%, 24.6% and 18.4% observed for the microspheres cross-linked by 3%, 5%, and 10% STPP. Following the initial burst, NGF released at a relatively slow rate from the first day to the end of the release process. Up to 91.0 ± 3.4% (1% STPP), 71.4 ± 2.9% (3% STPP), 62.2 ± 3.6% (5% STPP), and 50.4 ± 2.9% (10% STPP) of total NGF was released within 7 days (Table 2). A significant decrease was observed in finally cumulative release of NGF while the concentration of STPP increased from 1% to 10% ($p < 0.05$, Table 2).

3.3. In vitro bioactivity

The bioactivity of NGF released from NGF–CMSs was tested by PC12 cells which can differentiate into a neuronal phenotype with

Table 2
Burst release, Second and Third release rate, and Finally cumulative NGF release from NGF–CMSs.

STPP (%)	Burst release (%) (0–12 h)	Second release rate (%) (12 h–5 days)	Third release rate (%) (5–7 days)	Finally cumulative release (%) (0–7 days)
1%	45.5 ± 4.7	35.1 ± 3.2	10.4 ± 2.9	91.0 ± 3.4
3%	30.3 ± 2.9[a]	34.3 ± 2.8[a]	6.8 ± 3.1[a]	71.4 ± 2.9[a]
5%	24.6 ± 5.4[a,b]	31.1 ± 2.5[a,b]	6.5 ± 2.6[a,b]	62.2 ± 3.6[a,b]
10%	18.4 ± 4.7[a,b,c]	25.9 ± 2.1[a,b,c]	6.1 ± 1.6[a,b,c]	50.4 ± 2.9[a,b,c]

Data are expressed as mean ± SD.
[a] $p < 0.05$ when compared with 1% STPP.
[b] $p < 0.05$ when compared with 3% STPP.
[c] $p < 0.05$ when compared with 5% STPP.

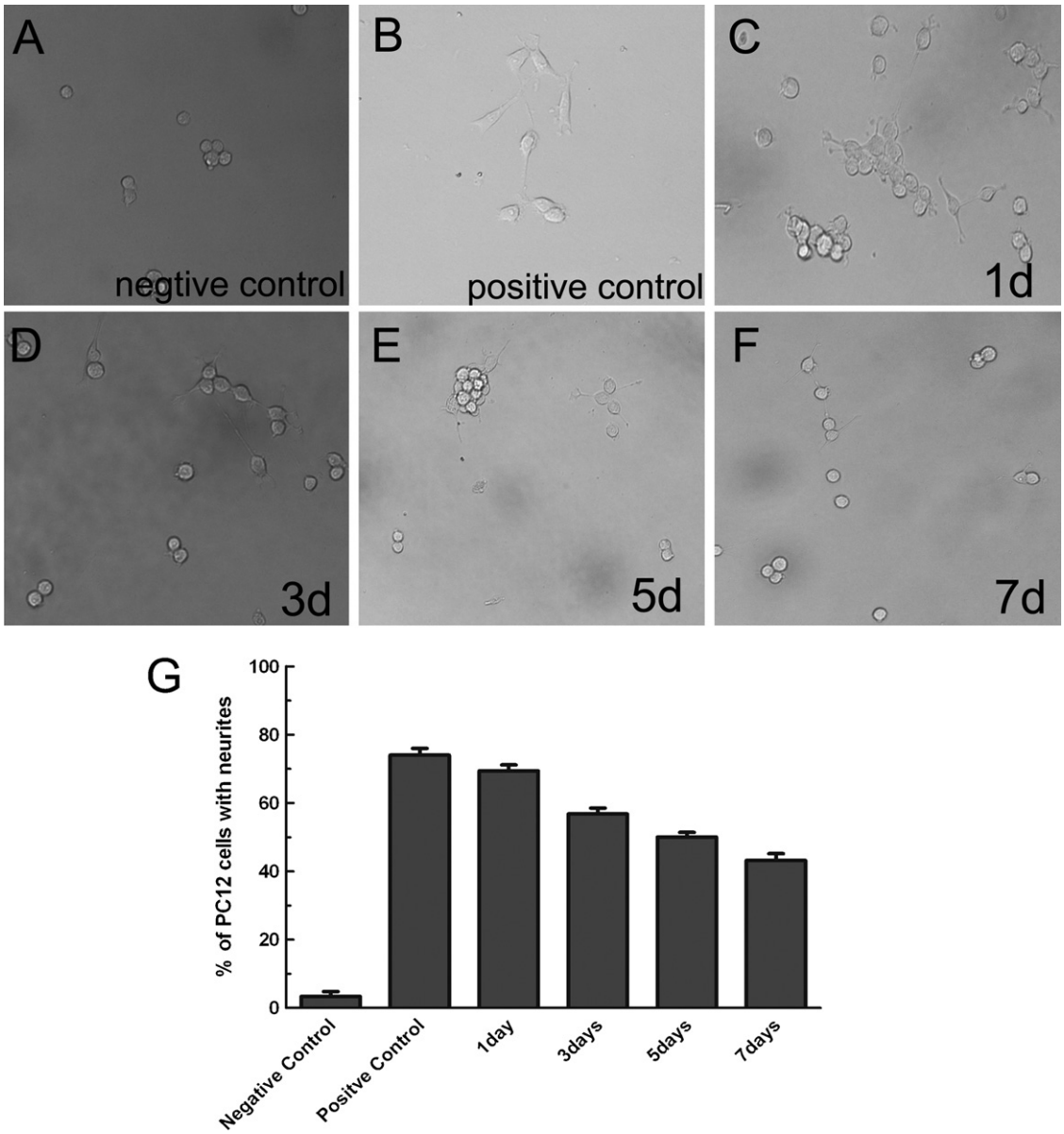

Fig. 5. Bioactivity of NGF released from NGF–CMSs. The morphology of PC12 cells which were incubated with culture medium without NGF (A, negative control), with 10 ng/ml NGF (B, positive control), with the release medium collected from NGF–CMSs incubation system at 1, 3, 5, and 7 days (C–F). The percentage of PC12 cells displaying neurite outgrowth was also shown (G).

axonal projections. PC12 cells cultured without NGF had round cell bodies and almost no neuritis (Fig. 5A). In contrast, PC12 cells showed positive response toward the NGF–CMSs release medium collected at different time points (1, 3, 5, and 7 days), indicating that the released NGF from NGF–CMSs maintained its bioactivity during the entire release testing period of 7 days (Fig. 5C–F). The percentage of PC12 cells with positive response was 69.4% when subjected to the release medium collected at 1 day, in contrast to 43.2% subjected to the medium collected at 7 days (Fig. 5G).

3.4. Cell viability (MTT) assay

MTT assay was used to study the viability of PC12 cells co-cultured with NGF–CMSs. The PC12 cells were incubated for 1, 3, 5, and 7 days in the control group (without NGF), NGF group (10 ng/ml NGF), and NGF–CMSs group (microspheres amount: 20 mg/ml, which contains 10 ng/ml NGF). After 1 day of culture, the cell viability in the three groups was in the similar range ($p > 0.05$, Fig. 6). At the end of the 3 days of culture, the cell viability in NGF group and NGF–CMSs group was significantly higher than that of the control group ($p < 0.05$, Fig. 6), whereas no statistical difference was observed between NGF and NGF–CMSs groups ($p > 0.05$, Fig. 6). After 5 days of culture, the cell viability in NGF–CMSs group was significantly higher than that in NGF group ($p < 0.05$, Fig. 6).

4. Discussion

Peripheral nerve regeneration is a dynamic process that needs activation of the intrinsic growth capacity of neurons and a permissive environment (Chen et al., 2007). Maintenance and survival of neurons are a prerequisite for early axonal regeneration after nerve injury (Sjoberg and Kanje, 1990). One approach toward promoting neuronal survival and regeneration is through the local application of exogenous NGF to the nerve injury site (Terenghi, 1999). The

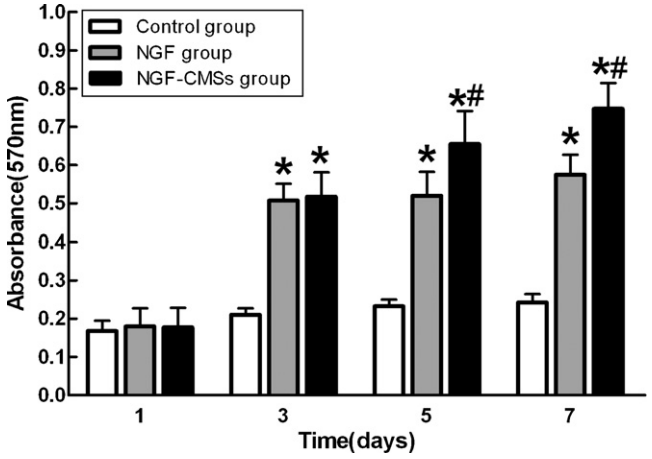

Fig. 6. The MTT assay results of the PC12 cells which were cultured for 1, 3, 5, and 7 days in the control group (without NGF), NGF group (10 ng/ml NGF), and NGF–CMSs group (microspheres amount: 20 mg/ml, which contains 10 ng/ml NGF). $^{*}p < 0.05$ when compared with control group; $^{\#}p < 0.05$ when compared with NGF group (10 ng/ml NGF).

present study shows that ionically cross-linked chitosan microspheres are capable of achieving controlled release of bioactive NGF. The encapsulation efficiency of NGF–CMSs decreased while the concentration of ionic cross-linking agent STPP increased. Further studies revealed that NGF could be released over 7 days in a controlled manner from NGF–CMSs, indicating that NGF–CMSs were effective in controlled release of NGF. In addition, *in vitro* bioactivity assay and MTT assay showed that the released NGF from NGF–CMSs was able to maintain the viability of PC12 cells, as well as promote their differentiation into neuronal phenotype.

Chitosan was selected in this study for developing microsphere-based delivery system because of its well-known biocompatibility, biodegradability, low toxicity and low cost (Illum et al., 2001; Vord et al., 2002; Khor and Lim, 2003). It has been reported that chitosan and chitosan-based materials have been shown to enhance neural cell adhesion, survival and neurite outgrowth of neurons (Yannas and Hill, 2004; Huang et al., 2010a,b). Thus, it is hypothesized that incorporation of NGF into chitosan microspheres has a dual function and could further promote neuronal differentiation and neurite outgrowth. In this study, we have demonstrated that NGF–CMSs have the potential to not only maintain PC12 cell viability, but also promote its differentiation and neurite outgrowth. However, further studies are needed to identify the possible beneficial effect of NGF–CMSs on nerve regeneration in *in vivo* studies.

Many approaches have been adopted to prolong the time frame and improve the release property of chitosan microspheres. During the preparation of chitosan microspheres, glutaraldehyde is one of the most widely used chemical cross-linking agents to achieve controlled release of targeted protein (Jameela and Jayakrishnan, 1995). However, it may compromise biocompatibility of chitosan microspheres and cause undesirable side effects such as loss of protein bioactivity by reacting with peptide and protein. To avoid the negative effects of chemical cross-linking agents, ionic cross-linking agents have been developed. STPP, a non-toxic and multivalent polyanions, has been widely used as an ionic cross-linking agent in the pharmaceutical industry. Under mild conditions, polyelectrolyte complex can be spontaneously formed by ionic interaction between positively charged amino groups of chitosan and multivalent negatively charged STPP molecules (Shu and Zhu, 2002; Desai and Park, 2005). This interaction could be controlled by the charge density of STPP and chitosan. Therefore, the concentration of STPP is a very important factor which can influence the properties of chitosan microspheres. As reported by Ko et al. (2002), the release behaviors of felodipine from STPP-chitosan microspheres can be affected by several factors such as molecular weight of chitosan, cross-linking agent and various pH levels. However, the information on the effect of different STPP concentrations on the properties of NGF embedded chitosan microspheres is limited in literature. In the present study, chitosan concentration (2.0% (w/v)), oil/water volume ratio (15:1), and cross-linking time (1 h) were chosen that were based on previously published data on preparation and optimization of chitosan microspheres by emulsion-ionic cross-linking method (Kim et al., 2003; Niu et al., 2009a,b). Different concentrations of STPP were used to optimize the property of NGF–CMSs and improve their applicability in the controlled delivery of protein drugs.

It is known that high encapsulation efficiency of proteins within a microsphere-based delivery system is of significant importance for controlled delivery. We studied the relationship between the STPP concentration and encapsulation efficiency of NGF. We found that the encapsulation efficiency and loading amount of NGF–CMSs decreased while the concentration of cross-linking agent STPP increased. Although this result would be attributed to a number of factors, the most important one might be as follows: NGF has isoelectric points of 9.0–9.35. Therefore, it carries positive charge in PBS (Ortega et al., 2001). During the preparation of chitosan microspheres, ionic cross-linking agent STPP may be firstly reacted with positively charged chitosan. However, if the concentration of STPP is added with excess, STPP might be further interacted with NGF, which will influence the incorporation of NGF into the chitosan matrix. In addition, we also studied the relationship between the STPP concentration and mean size of NGF–CMSs. We found that mean size of NGF–CMSs increased while the STPP concentration increased. This observation might be explained as follows: the preparation of chitosan microspheres were realized through polyelectrolyte complex formation which are formed by ionic interaction between positively charged amino groups of chitosan and multivalent negatively charged STPP molecules (Shu and Zhu, 2002; Desai and Park, 2005). This interaction could be controlled by the charge density of STPP and chitosan. When STPP concentration increased, small chitosan microspheres might be fused into bigger ones. Therefore, the size of microspheres increased when STPP concentration increased.

The sustained release of NGF from NGF–CMSs was monitored over 7 days. In the present study, STPP concentration played an important role in the release properties of NGF–CMSs during the preparation process. The amount of NGF released from NGF–CMSs decreased when the concentration of STPP increased. It might be due to the high cross-linking density which prevents the swelling of the microspheres and further reducing NGF quantities released. Total release of encapsulated NGF from NGF–CMSs did not take place. This suggests that positively charged NGF was retained in the microspheres by the electrostatic interactions with negatively charged STPP (Kim et al., 2003). In addition, three stages were found during the release process of NGF from NGF–CMSs. In the initial burst stage (0–12 h), approximately 18–45% of total encapsulated NGF was released from NGF–CMSs. This indicates that NGF placed on the surface of the microspheres was released rapidly. Similar burst stage has been reported for the release of different drugs from chitosan microspheres (Huang et al., 2003; Corrigan et al., 2006). After the burst stage, the second (12 h–5 days) and third (5–7 days) stages were followed in a slow manner. These processes were realized by NGF diffusion through pores and channels in the microspheres and erosion controlled release by microspheres biodegradation. Kim et al. (2003) have reported similar biphasic drug release from chitosan microspheres. They conclude that the initial burst stage followed by slower ones at remaining days is highly governed by drug diffusion. In the present study, the microspheres sopped up and enlarged rapidly which contributed

greatly to the initial burst release of NGF from the surface of microspheres. As swelling was counterpoise, the release of NGF was slowed down in the second and third stages and the NGF release in these two stages would be determined by NGF diffusion and the microspheres biodegradation. Taking the higher encapsulation efficiency and the controlled release kinetics *in vitro* into consideration, the microspheres cross-linked with 3% STPP was selected for further study.

The bioactivity of NGF released from NGF–CMSs was assessed by observing the differentiation of PC12 cells and evaluating the cell viability. PC12 cells have been widely used as a model for neuronal differentiation in response to exogenous signals such as growth factors and neurotransmitters (Sofroniew et al., 2001). Therefore, we cultured PC12 cells to demonstrate the bioactivity of NGF released from NGF–CMSs. In the study of the differentiation of PC12 cells, we found that supernatant which was obtained from NGF released solution at different time points showed a positive effect on PC12 cells when it was added into PC12 cells culture medium. This can be explained by the binding of bioactive NGF released from NGF–CMSs to receptors on PC12 cells, such as Tyrosine kinase receptor A (TrkA), which mediates NGF-induced cell differentiation and survival (Zhang et al., 2000). The bioactive protection of released NGF from NGF–CMSs may be ascribed partly by co-encapsulation of bovine serum albumin (BSA). BSA may be a carrier for NGF and it can protect the bioactivity of NGF released from the microsphere. In the present study, NGF–CMSs maintained the viability of PC12 cells compared with NGF added to culture medium at the end of 7 days of culture. There may be two possible mechanisms to explain it. Firstly, NGF–CMSs as a growth factor reservoir may play an important role in stabilizing the bioactivity of NGF. In contrast, NGF added to cell culture medium may lose bioactivity rapidly (Fu et al., 2000). Secondly, bioactive NGF, as well as chitosan and its biodegradation products may have a synergistic effect on maintaining cell viability. In brief, bioactive NGF continued to be released over 7 days in a controlled manner, indicating that chitosan microspheres mediated NGF delivery is a viable option for controlled release of bioactive NGF.

5. Conclusion

In this research, we have prepared NGF loaded chitosan microspheres with STPP as non-toxic and mild cross-linking agent using the emulsion-ionic cross-linking method. The microspheres had a rough surface and proper size distribution. High encapsulation efficiency was obtained. The microspheres were able to release NGF in a controlled manner for at least 7 days *in vitro*. In addition, the released NGF from NGF–CMSs was capable of maintaining the viability of PC12 cells, as well as promoting their differentiation, suggesting that NGF–CMSs are capable of releasing bioactive NGF over 7 days. The NGF–CMSs prepared in the present study have potential applications in nerve tissue engineering and regenerative medicine.

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