Original Paper

Dual Delivery of bFGF- and NGF-Binding Coacervate Confers Neuroprotection by Promoting Neuronal Proliferation

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Key Words

Basic fibroblast growth factor (bFGF) • Nerve growth factor (NGF) • [PEAD:heparin] coacervate • Neurodegenerative diseases • PC12 cells • SH-SY5Y cells

Abstract

Background/Aims: Basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) are essential for proper development, survival, growth, and maintenance of neurons in the central and peripheral nervous systems. However, because bFGF and NGF have short half-life and rapid diffusion rate, they have limited clinical efficacy. Thus, there is an urgent need to develop an effective delivery system to protect bFGF and NGF from proteolysis while maintaining their normal bioactivities. **Methods:** To more efficiently deliver bFGF and NGF, we used a coacervate (synthesized with heparin and a biodegradable polycation at mass ratio of 500:100). The maximal package loads of GFs in coacervate were determined by Western Blotting; release efficiency of bFGF and NGF was measured by ELISA. Additionally, we evaluated the effect of bFGF and NGF on the viability, survival, and proliferation of neurons by MTT assay, BrdU cell proliferation, and calcein staining. **Results:** Our coacervate incorporated bFGF and NGF and continuously released them for at least three weeks. This enhanced the growth and proliferation of PC12 cells and SH-SY5Y cells. Moreover, co-delivery of bFGF and NGF using coacervate was more neuroprotective than free application of both factors or coacervate delivery of

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each GF separately. **Conclusions:** Dual delivery of bFGF and NGF binding coacervate was neuroprotective via stimulating the growth and proliferation of neurons.

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Introduction

Neurodegenerative diseases are a major socioeconomic burden and lead to unimaginable misery for millions of sufferers and their families around the world. With an increasing ageing population, the number of neurodegenerative diseases is expected to rise even further, prompting an urgent need for the development of rational and effective therapeutic strategies that can reverse or slow the degenerative process. Stem cell therapy, growth factors, and gene therapy are novel treatments that might prolong survival and delay progression of symptoms [1-4]. Growth factor treatment is one of the most promising therapies for neurodegenerative diseases, due to their great potential for neurorestoration and neuroprotection.

Within the family of neurotrophic factors, nerve growth factor (NGF) stimulates the survival and maturation of developing neurons in the peripheral nervous system and protects neurons in the degenerating mammalian brain [5-8]. It has been used as a therapeutic agent for the restoration and maintenance of neuronal function in both basic and clinical studies. Basic fibroblast growth factor (bFGF) is highly expressed in the nervous system and exerts multiple roles supporting the survival and growth of cultured neurons and neural stem cells [8-10]. Although bFGF and NGF have distinctive neuroprotective properties, their short half-life and rapid diffusion rate seriously hinders their clinical applicability. Thus, the development of a useful delivery system that not only protects the GFs from proteolytic degradation and controls their spatiotemporal release, but also avoids side effects from high concentrations of GFs for body would greatly improve their clinical efficacy.

[PEAD:heparin] coacervate is formed by a polycation, poly ethylene arginyl aspartate diglyceride (PEAD), and heparin [11]. PEAD is a biodegradable polycation with high biocompatibility and high charge density, which strongly binds to heparin and consequently is capable of incorporating growth factors with high efficiency [11]. Previous studies have demonstrated that [PEAD:heparin] coacervate controls the release of growth factors for over 30 days in a nearly linear fashion, can maintain the bioactivity of bFGF, and can increase the bioactivity of NGF [8], indicating that [PEAD:heparin] coacervate may be an optimal delivery system for growth factors for the treatment of neurodegenerative diseases.

Although the delivery of a single GF, bFGF or NGF, has shown promising therapeutic potency for neurodegenerative diseases, co-delivery of multiple GFs may be more successful in therapy. In this study, we co-delivered bFGF and NGF via [PEAD:heparin] coacervate and examined the effect of controlled co-release of bFGF and NGF on PC12 cells and SH-SY5Y cells. We hypothesized that co-delivery of bFGF and NGF would have a stronger and more robust neuroprotective effect than that of individual GF coacervates and the co-administration of free GFs.

Materials and Methods

Reagents and antibodies

Recombinant human bFGF was purchased from Grost (Grost Biotechnology, Zhejiang, China). NGF and heparin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). PEAD was a gift from the University of Pittsburgh. NGF and bFGF enzyme linked immunosorbent assay (ELISA) kits were purchased from Elabscience Biotechnology. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA).

[PEAD:heparin] coacervate synthesis and maximal package loads of GFs

Poly ethylene arginylaspartate diglyceride (PEAD) was synthesized as previously described [11]. PEAD, heparin, NGF and bFGF were serially dissolved in 0.9% normal saline to obtain 10 mg.mL⁻¹ solutions

and sterilized using 0.22 μ m Millipore filter. We used a PEAD:heparin:GF with mass ratio of 500:100:1 to achieve maximal coacervation. The bFGF was loaded into the coacervate at doses of 50ng, 100ng and 10 μ g respectively; the NGF was loaded at doses of 1 μ g, 2 μ g and 10 μ g respectively. After 2 h, the solution was then centrifuged at 12000g for 10 min to pellet the coacervate at 4°C. The supernatant was aspirated and stored, and pellet was re-suspended using loading buffer to detect the maximal package loads of GFs in coacervate by Western blotting.

Western blotting

To detect the maximal package loads of GFs, protein concentrations were quantified using a BCA Protein Assay Kit (Thermo, Rockford, IL, USA). The equivalent of total protein was loaded onto SDS-PAGE, transferred to PVDF membrane (Bio-Rad), and blocked with 5% non-fat-milk in TBS with 0.05% Tween 20 (TBST) for 45min. Primary antibodies were incubated overnight at 4°C. The membranes were washed with TBST for 3 times and incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000) for 4h at room temperature. Signals were visualized using the ChemiDoc™ XRS +Imaging System (Bio-Rad).

Growth factors release assay

On day 1, 4, 7, 14, 21, 28 and 35, PEAD: heparin: GF coacervates with maximal package loads of bFGF and NGF were gently mixed and centrifuged at 12, 000 g for 10 min. Then, the supernatant was collected to detect the amount of released GFs via ELISA kit. Standards containing 200 ng free-form GFs were used to determine the percent release. The samples and standards were reacted with assay buffer. The absorbance of the supernatant was recorded by a SynergyMX plate reader at 450/540 nm.

Cell viability measurements

MTT assay was used to determine cell viability and the optimal concentration of bFGF and NGF. Briefly, cells were plated at a density of 8000 per well in a 96-well plate. After 6 h, the cells were washed with PBS and cultured in serum-free medium. Then, the cells were treated with bFGF and NGF at doses of 50nM, 100nM, 200nM and 400nM respectively. After 24 h incubation, 20 μ L MTT buffer was added to each well. The absorbance was recorded by a SynergyMX plate reader at 490 nm.

Detection of cell growth status

PC12 cells and SH-SY5Y cells were plated at a density of 8000 per well in a 96-well plate. 6h after seeding, group-specific media was added and 8 groups were used with 5 wells per group: basal media, blank coacervate, free bFGF, free NGF, free bFGF+NGF, bFGF coacervate, NGF coacervate, and bFGF+NGF coacervate. Each GF was added at an optimal concentration according to MTT assay results. Cell growth status was observed and captured under an inverted microscope (Nikon ECLIPSE Ti-S). The area of cell number was quantitatively analyzed.

The proliferation of neuronal cells and live cell

Similar culture conditions were used for detecting the level of cell proliferation and performing live cell count assays. PC12 cells and SH-SY5Y cells were labeled with calcein AM for 2 h before seeding cells in 100 μ L media per well in a 96-well plate. 6 h after seeding, group-specific media was added and 8 groups were used with 5 wells per group: basal media, blank coacervate, free bFGF, free NGF, free bFGF+NGF, bFGF coacervate, NGF coacervate, and bFGF+NGF coacervate. Each GF was added at an optimal concentration. For the BrdU cell proliferation assay, the plate was incubated at 37°C for 16 h, then 20 μ L of BrdU label was added to each well and incubated for 4 h. The proliferation assay protocol was then followed according to the kit's instruction manual. After the addition of stop solution, the absorbance at 450/540 nm was recorded by a SynergyMX plate reader and normalized to the basal media control. For the live cell count assay, the plate was incubated at 37°C for 3 d, then cells were observed using a fluorescence microscope. The number of cells was determined by manually counting the cells in a 0.67 mm² field in the center of the well for 5 wells per group. Fluorescent images of cells were taken of 4 mm² fields.

Statistical analysis

Data is presented as the mean $\pm$ SD. Statistical differences were evaluated using One-way analysis of variance (ANOVA) followed by Tukey's test with GraphPad Prism 5 software. Differences were suggested to be statistically significant when $P < 0.05$.

Results

Loads and release efficiency of bFGF and NGF in [PEAD:heparin] coacervate

It had been demonstrated that maximal coacervation occurs at a mass ratio of 500:100:1 PEAD:heparin:GF [12]. In this study, we utilized PEAD:heparin:GF coacervate with this mass ratio and observed that the coacervate became turbid and precipitated immediately with bound GFs to the coacervate (Fig. 1A). After 24 h, the coacervate became pellucid and settled on the bottom of the tube (Fig. 1A). The maximal loads of bFGF and NGF in [PEAD:heparin] coacervate were detected by Western blotting. 100ng, 200ng and 10 μ g of bFGF and 1 μ g, 2 μ g and 10 μ g of NGF were bound to coacervate. The maximal loading capacity of each was found to be 10 μ g (Fig. 1B). To further investigate the release efficiency of GFs that bound with coacervate, we measured the release amount of bFGF and NGF by ELISA on days 1, 4, 7, 14, 21, 28 and 35. NGF and bFGF were immediately released on day 1, and then sustained from day 1 to day 21. Afterwards, both were released slower and gradually trended to stabilize. By day 35, the total release of NGF and bFGF were approximately 60% and 30%, respectively. Taken together, this not only demonstrated that coacervate loading with GFs contributes to their slow release, but also indicated that the release efficiency of NGF was higher than that of bFGF in coacervate.

Determining the optimal protective concentration of bFGF and NGF on PC12 cells and SH-SY5Y cells

To test the optimal protective concentration of bFGF and NGF on PC12 cells and SH-SY5Y cells, we assessed the cell viability of PC12 cells and SH-SY5Y cells after treatment with different doses of each GF. We found 100 ng/mL to be the optimal protective concentration of bFGF on PC12 cells and SH-SY5Y cells (Fig. 2). Additionally, 100ng/mL and 200ng/mL of NGF were the optimal protective concentrations for PC12 cells and SH-SY5Y cells (Fig. 2).

Fig. 1. Properties of [PEAD:heparin] coacervate binding with bFGF or NGF. (A) Preparation of [PEAD:heparin] coacervate. (B) Western blotting analysis of the loading efficiency of bFGF and NGF into coacervate. S: bFGF or NGF in supernatant after centrifugation. C: bFGF or NGF in settled coacervates. L: total amount of bFGF or NGF in loading solution. (C) Release efficiency of bFGF and NGF in [PEAD:heparin] coacervate. (n= 3 per group).

Fig. 2. The optimal protective concentration of bFGF and NGF on PC12 cells and SH-SY5Y cells. (A) The optimal protective concentration of bFGF and NGF on PC12 cells. (B) The optimal protective concentration of bFGF and NGF on SH-SY5Y cells. *P<0.05 vs. the other groups.

bFGF+NGF coacervate promotes the growth of PC12 cells and SH-SY5Y cells

To assess how bFGF and NGF affect neuronal cell growth, PC12 cells were cultured in serum-free culture medium and each GF was applied separately or together in free-form or coacervate-bound. PC12 cells in serum-free culture medium had fewer cell number and abnormal morphology with karyopyknotic nuclei and shortened axons (Fig. 3A, B). Treatment with bFGF and NGF together in free-form recovered PC12 cell growth better than each GF alone. Moreover, each GF alone delivered by coacervate also significantly promoted PC12 cells growth when compared to free GF delivery. Importantly, compared to the other groups, dual delivery of bFGF and NGF in coacervate was the most beneficial to PC12 cells growth as evidenced by increased cell number and normal morphology (Fig. 3A, B). Consistent with our findings in PC12 cells, dual delivery of bFGF and NGF bound to coacervate also significantly promoted SH-SY5Y cells growth (Fig. 4A, B).

bFGF+NGF coacervate induces the proliferation of PC12 cells and SH-SY5Y cells

In this study, we further investigated the effect of bFGF and NGF on the proliferation of PC12 cells. PC12 cells were treated with each GF separately or together, in free-form or coacervate bound. 1 day after treatment, Free-form dual delivery of GFs and each GF alone delivered by coacervate significantly induced greater proliferation compared to each GF alone. Furthermore, dual delivery of bFGF and NGF with coacervate had a stronger effect compared to the other groups (Fig. 5 A). Calcein staining was used to verify the cell proliferation beyond 1 day, and cells were still viable after 3 days culture. Consistent with these results, co-delivery of bFGF and NGF bound to coacervate exerted a stronger proliferative effect and induced more live cells compared to the other group (Fig. 5 B, C). Additionally, the bFGF+NGF coacervate could significantly induce the proliferation of SH-SY5Y cells (Fig. 6 A, B and C). Taken together, these results demonstrated that co-delivery of bFGF and NGF with coacervate had the greatest potential for promoting the proliferation of neuronal cells.

Fig. 3. bFGF+NGF coacervate promotes the cell growth of PC12 cells. (A) Quantitative analysis of the number of PC12 cells. (n= 8 per group). *P<0.05 indicated Free bFGF+NGF, bFGF+coacervate and NGF+coacervate vs. basal media, blank coacervate, free bFGF and free NGF group; #P<0.05 indicated bFGF+NGF+coacervate vs. the other groups. (B) The morphology of PC12 cells after treatment.

Fig. 4. bFGF+NGF coacervate promotes the cell growth of SH-SY5Y cells. (A) Quantitative analysis of the number of SH-SY5Y cells. (n= 8 per group). *P<0.05 indicated Free bFGF+NGF, bFGF+coacervate and NGF+coacervate vs. basal media, blank coacervate, free bFGF and free NGF group; #P<0.05 indicated bFGF+NGF+coacervate vs. the other groups. (B) The morphology of SH-SY5Y cells after treatment.

Fig. 5. bFGF+NGF coacervate induces the proliferation of PC12 cells. (A) The proliferation level of PC12 cells after treatment with free GFs or coacervate delivery of GFs. (B) Live PC12 cells number was quantified after 3 days incubation over 0.67 mm² fields in 3 wells per group. (C) Microscope images of calcein-stained PC12 cells. *P<0.05 indicated Free bFGF+NGF, bFGF+coacervate and NGF+coacervate vs. basal medial, blank coacervate, free bFGF and free NGF group; #P<0.05 indicated bFGF+NGF+coacervate vs. the other groups.

Fig. 6. bFGF+NGF coacervate induces the proliferation of SH-SY5Y cells. (A) The proliferation level of SH-SY5Y cells after treatment with free GFs or coacervate delivery of GFs. (B) Live SH-SY5Y cells number was quantified after 3 days incubation over 0.67 mm² fields in 3 wells per group. (C) Microscope images of calcein-stained SH-SY5Y cells. *P<0.05 indicated Free bFGF+NGF, bFGF+coacervate and NGF+coacervate vs. basal medial, blank coacervate, free bFGF and free NGF group; #P<0.05 indicated bFGF+NGF+coacervate vs. the other groups.

Discussion

Neurodegenerative diseases are a more and more serious problem for the aging population in the world. They are characterized by progressive loss of neuronal function in defined regions of the nervous system. Elevated cellular stress, mitochondrial dysfunction, synapse loss, and neuronal apoptosis are the main physiological symptoms of neurodegenerative diseases [13]. No broadly effective strategies have been developed recently to reverse or halt the progression of these diseases in patients. bFGF and NGF have been extensively shown to promote the survival and maintenance of neuronal function in neurological diseases [14, 15]. A deficiency of these two GFs may cause neuron susceptibility to injury and death [16, 17], suggesting that molecular therapy with exogenous bFGF and NGF may be a novel and challenging therapeutic strategy for neurodegenerative diseases.

However, the application of these GFs, including NGF and bFGF, in clinical therapy is greatly limited by their short half-life. Without efficient delivery systems, GFs degrade quickly and lose their bioactivities, which potentially lead to harmful effects when injected at high concentrations for effective therapy. Moreover, GFs diffuses broadly circulate throughout the body, which leads to adverse effects that can be intolerable. Previous studies reported that NGF led to pain and weight loss by stimulating nociceptive and hypothalamic neurons,

respectively [18, 19]. Developing a controlled and useful delivery system that can preserve their processibility and biocompatibility is particularly attractive for their biomedical application. In this study, we utilized [PEAD:heparin] coacervate as a delivery system to deliver NGF and bFGF, and further confirmed the effects of bFGF and NGF on growth and proliferation of neuronal cells.

[PEAD:heparin] coacervate was formed by a polycation, poly(ethylene arginyl aspartate diglyceride) (PEAD) and heparin that can control the release of heparin-binding GFs [20]. The coacervate maintains the native properties and function of heparin through the direct, ionic interactions with PEAD [21], which not only improves GF loading efficiency and maintains their bioactivity, but also well controls the release of GFs to target tissues [20]. Thus, coacervate had been widely used to spatially and temporally control the release of GFs [22-25].

Wang *et al.* demonstrated that the molecular weight of PEAD and/or heparin, the charge density of PEAD, and the [PEAD:heparin] mass ratio affected the delivery function of [PEAD:heparin] coacervate. Moreover, a PEAD:heparin:GF mass ratio of 500:100:1 achieved maximal coacervation [21]. In this study, we developed [PEAD:heparin] coacervate with a PEAD: heparin: GF mass ratio of 500:100:1 to deliver bFGF and NGF. Maximal loading was reached with 10 μ g of bFGF and NGF, and GFs-binding with coacervates sustained their release from day 1 to day 21 (Fig. 1B and C), indicating that coacervate slows the release of GFs. Additionally, with the different dissociation constant (kd) of bFGF (2 nM) and NGF (600 nM), the release efficiency of NGF(60%) was higher than that of bFGF(30%) in coacervate(Fig. 1C), consistent with our previous study [8].

PC12 cells and SH-SY5Y cells are extensively used as neuronal cell models *in vitro* [26-28]. In this study, PC12 cells and SH-SY5Y cells were used as a model system to detect the neuroprotective effects of bFGF and NGF. Both bFGF and NGF are known to promote an appropriate environment for neurogenesis of stem cells as well as nerve regeneration [29, 30]. We found that bFGF and NGF together in free-form and each GF alone incorporated in coacervate significantly increased the growth and proliferation of PC12 cells and SH-SY5Y cells compared to each GF alone. This study in conjunction with previous studies have demonstrated that combined treatment with NGF and bFGF has a greater potential to promote the proliferation and differentiation of neural stem cells and nerve regeneration [8, 31]. Thus, dual delivery of bFGF and NGF using coacervate greatly increased the growth and proliferation of neuronal cells when compared to all the other groups, suggesting that controlled dual-delivery of bFGF and NGF using [PEAD:heparin] coacervate does not hinder the beneficial effects of bFGF and NGF, but rather improves it when compared to free application of both factors or coacervate delivery of each GF separately.

Conclusion

Our results further confirm the neuroprotective effects of NGF and bFGF on the growth and proliferation of neuronal cell types. Moreover, co-delivery of bFGF and NGF using coacervate displayed the most robust neuroprotective effect individual GF coacervates or co-administration of free GFs. These findings highlight that dual bFGF and NGF-incorporated coacervate may be more effective molecular therapeutics for neurodegenerative diseases.

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Disclosure Statement

The authors declare to have no competing interests.

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