

Preparation and *in vitro* activity of controlled release microspheres incorporating bFGF

SHEN Bin 沈彬, PEI Fu-xing 裴福兴*, DUAN Hong 段宏, CHEN Jian 陈坚 and MU Jian-xiong 牟建雄

Objective: To study the preparative method of controlled release microspheres incorporating basic fibroblast growth factor (bFGF) and the bioactivities of bFGF, which were released from bFGF microspheres, on the cultured Schwann cells.

Methods: bFGF was microcapsulated with the multiple emulsion encapsulative method using polylactic-co-glycolic acid (PLGA) as coating material. Its morphology, particle size distribution, drug loading, enveloping rate and *in vitro* release property were studied. The cultured Schwann cells were grouped according to the different ingredients being added to the culture medium of bFGF group or bFGF-PLGA group. Then the cytometry, cytoactivity detection and mitotic cycle analysis of Schwann cells were performed.

Results: The morphology and the particle size distribution of the bFGF-PLGA microspheres were even and good. The drug loading and enveloping rate of microspheres were $(27.18 \times 10^{-3})\% \pm (0.51 \times 10^{-3})\%$ and $66.43\% \pm 1.24\%$. The release property of microspheres *in vitro* was good and the overall release rate was 72.47% in 11 days. The *in vitro* cellular study showed that: at the first

2 days of plate culture, the cell number and viability of the bFGF group were statistically higher than the bFGF-PLGA group; at the 3rd and 4th days of plate culture, the cell number and viability of bFGF and bFGF-PLGA groups showed no difference; at the 6th and 8th days of the plate culture, the cell number and viability of the bFGF-PLGA group were statistically higher than the bFGF group. By flow cytometry examination, at the 2nd day of plate culture, the G2/M + S percentage of bFGF group was statistically higher than the bFGF-PLGA group, at the 4th and 8th days of plate culture, the G2/M + S percentage of the bFGF-PLGA group was statistically higher than the bFGF group.

Conclusions: It is practical to prepare the bFGF-PLGA microspheres with the multiple emulsion encapsulative method. bFGF-PLGA microspheres can preserve the bioactivities of bFGF effectively and promote the proliferation of Schwann cells in a long period because of the controlled release of bFGF from the microspheres.

Key words: Fibroblast growth factors; Microspheres; Schwann cells

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Basic fibroblast growth factor (bFGF) has been proved to have mitogenic and differentiation-promoting effect on neuroectodermal cells, and it has great applicable potentials in the research field of nerve regeneration.^{1,2} However, because of the instability in the heat and acid environment, along with a short half-life time ($T_{1/2}$) of 3-5 minutes *in vivo*, bFGF cannot satisfy the need for clinical applications either universally or locally. So it is meaningful to find out a controlled release agent of bFGF by which to maintain a prolonged effective drug concentration and improve nerve regeneration.^{3,4}

In this study, polylactic-co-glycolic acid (PLGA) was used as the coating material, and a multi-emulsion encapsulating mechanism was used in the preparation of controlled release microspheres incorporating bFGF. Its morphology, particle size distribution, drug loading, enveloping rate and *in vitro* release property were studied. In addition, cytometry, cytoactivity detection and mitotic cycle analysis were performed in order to achieve a preliminary idea about the bioactivity of the bFGF being released from the controlled release microspheres through adding the bFGF microspheres into the culture medium of the Schwann cells.

METHODS

Reagent and equipment

Freeze-dried bFGF powder (EssexBio, China), Polylactic-co-glycolic acid copolymer (Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Mv 12 000, 50; 50), Rabbit polyclonal

Department of Orthopaedic Surgery, West China Hospital of Sichuan University, Chengdu 610041, China (Shen B, Pei FX, Duan H, Chen J and Mu JX)

* Corresponding author; Tel: 13808078204 E-mail: peifuxing@vip.163.com

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antibody against bovine bFGF (Sigma, USA), Goat antibody against rabbit IgG marked with horseradish peroxidase enzyme (Beijing Zhongshan Biotech Ltd Co., China), Polyclonal antibody against S-100 protein (Beijing Zhongshan Biotech Ltd Co., China), Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA), Type-II Collagenase (Sigma, USA), Trypsin (Sigma, USA), Collagen (Sigma, USA), Methylthiazolyl tetrazolium (MTT Sigma, USA).

L8-80M speeding refrigeration centrifuge (USA), AMRAY electronic scanning microscopy (USA), Malvern mastersizer 2000 laser particle size distribution tester (USA), Lyophilizer (Savant Modulyo, USA), Bio-RAD Model 550 photodensitometer (USA), Flow cytometer (Elite SP, USA), Class II A/B3 biological safety cabinet (USA), CO₂ gas incubator (Sanyo, Japan), Refrigeration centrifuge (Megafuge 1. OR, USA).

Preparation of bFGF-PLGA controlled release microspheres

According to the optimized prescription obtained from the preliminary test, PLGA was dissolved by the dichloromethane solution. Once completely dissolved, the bFGF solution was added and the mixture was homogenized by ultrasonic wave into emulsion. 6% polyvinyl alcohol (PVOH) solution was used as the aqueous phase dispersed medium, whose inorganic salt concentration was 2%. 2 ml PVOH solution was then mixed into the emulsion. After completely stirred, the emulsion was added into 8 ml polyvinyl alcohol (PVOH) solution of the same concentration, stirred by magnetic force under ambient temperature until the complete volatilization of the dichloromethane, and the colloid solution of bFGF-PLGA controlled release microspheres was then obtained. Proper amount of 1% lactose as frame materials was added into the microsphere solution which was conserved under freeze-dried circumstance.

Morphology and particle size distribution of bFGF-PLGA microspheres

Electronic scanning microscopy was used to observe the morphology of bFGF-PLGA microspheres. Malvern laser particle size distribution tester was used to determine the particle size of the bFGF-PLGA microspheres. The size distribution of the bFGF-PLGA microspheres was calculated according to the Chinese

Dispensary 2000: $\text{size distribution} = (D90 - D10) / D50$.

Examination of drug loading and enveloping rate of the bFGF-PLGA microspheres

The enzyme linked immunosorbent assay (ELISA) was used to examine the absorbency (*A* value) of the standard bFGF solution. The *A* value was used for the logarithm linear regression of the standard bFGF solution and the equation was established. Certain amount of newly prepared bFGF-PLGA microspheres was obtained and centrifuged at 40 000 r/min under 4°C for 2 hours. The ELISA was used to determine the *A* value of the supernatant fluid and the floating drug load (the drug load in the liquid medium) was calculated according to the regression equation. The drug loading and enveloping rate were then obtained according to the formulations.

In vitro drug release of bFGF-PLGA microspheres

According to the drug loading, bFGF-PLGA microspheres which contain 1.5 μg bFGF were added into centrifugal tubes (*n* = 3). Then 2 ml normal saline was added into each tube and oscillated with constant temperature and speed (37°C and 20 r/min). The concentrations of bFGF in the supernatant fluid from the centrifuge tubes were measured at 12, 24, 36, 48, 60, 72, 84 hours and 5, 6, 7, 8, 9, 10, 11 days respectively. The original volumes were restored after sampling. The bFGF-PLGA microspheres releasing curve was drawn according to the accumulated release rate and time data.

Cultivation, identification and grouping of Schwann cells

Primary Schwann cells, which were obtained from bilateral sciatic nerves of newborn New Zealand rabbits, were cultivated using a combination of enzymic digestion and repeated attachment method. The cultivated Schwann cells were identified by immunohistochemistry staining using anti-S-100-protein polyclonal antibody and then subcultured for the subsequent use. Experiment grouping: Group A contained 10% calf serum and DMEM with 50 ng/ml bFGF; Group B contained 10% calf serum and DMEM with bFGF-PLGA microspheres which contain 50 ng/ml bFGF.

Effect of bFGF-PLGA microspheres on the cytometry, cytoactivity and mitotic cycle of Schwann cells

The 3rd subcultivated generation of Schwann cells with the concentration of 1×10^4 /ml were inoculated into a 24-well plate, 1 ml in each pore. When the cells reached an identical level of growth, different culture fluids were exchanged according to different groups. Cell samples of 2 wells in each group were collected at the 1st, 2nd, 3rd, 4th, 6th, 8th days and the cell numbers were calculated by haemocytometer, which were repeated three times.

The 3rd subcultivated generation of Schwann cells with the concentration of 1×10^4 /ml were inoculated into a 96-well plate, 200 μ l in each pore. When the cells reached an identical level of growth, different culture fluids were exchanged according to different groups. Cell samples of 4 wells in each group were collected at the 1st, 2nd, 3rd, 4th, 6th, 8th days. According to the regulation of MTT method, methylthiazolyl tetrazolium and dimethyl sulfoxide were added 4 hours before the collection, and ELISA reader was used to examine the absorbance (A value) of each well at 490 nm wave length. This procedure was repeated three times.

The 3rd subcultivated generation of Schwann cells with the concentration of 4×10^4 /ml were inoculated into a 6-well plate, 1 ml in each pore. When the cells reached an identical level of growth, different culture fluids were exchanged according to different groups. Cell samples of 2 wells in each group were collected on the 2nd, 4th, 8th days. Cell mitotic cycle was examined by flow cytometer, and the percentages of G2/M + S were calculated. This procedure was repeated three times.

Statistical analysis

The results were demonstrated in terms of mean \pm standard deviation ($\bar{x} \pm s$). Statistical analysis was done by using SPSS software. $P < 0.05$ is considered to be statistically significant. Student's *t*-test was used for the comparison of two groups and ANOVA was used for the comparison of multiple groups.

RESULTS

Morphological observation of bFGF-PLGA microspheres

The bFGF-PLGA microspheres had smooth round surfaces, uniform partical size and no adhesion were observed. The mean partical size was $(1.552 \pm 0.015) \mu\text{m}$ and the mean size distribution was 1.310 ± 0.010 , which showed that the particle distribution was narrow and their sizes were uniform (Fig. 1).

Drug loading and enveloping rate of bFGF-PLGA microspheres

The regression equation obtained from the standard bFGF solution was $C = 1127.81 \times A - 20.72$, $r = 0.9697$. The drug loading and enveloping rate of bFGF-PLGA microspheres were $(27.18 \times 10^{-3}) \pm (0.51 \times 10^{-3})\%$ and $(66.43 \pm 1.24)\%$ respectively (Table 1).

Table 1. Drug-loading and enveloping rate of bFGF-PLGA microspheres

Batch No.	Drug loading (10^{-3})	Enveloping rate (%)
1	26.42	65.67
2	27.04	66.32
3	28.08	67.30

In vitro drug release of bFGF-PLGA microspheres

Within the 11 days period, bFGF were released from the microspheres constantly and cumulatively. The concentration of bFGF was in a range of 71.47-89.81 ng/ml while the overall releasing rate reached 72.47% at the 11th day.

Cultivation and identification of Schwann cells

The typical Schwann cells are fusiform with two slender prominencies at the two poles. The nucleus is elliptical, seating at one side of the cell body. Immunohistochemistry staining of polyclonal antibody of S-100 protein showed a diffuse positive reaction in the cytoplasm (Figs. 2, 3).

Effect of bFGF-PLGA microspheres on the cytometry of Schwann cells

The number of cultured Schwann cells at different time points showed that: 1, 2 days after plate culture, the number of Schwann cells of the bFGF group was statistically more than that of bFGF-PLGA group

($P < 0.05$); 3, 4 days after plate culture, the number of bFGF group and bFGF-PLGA group showed no difference ($P > 0.05$); 6, 8 days after plate culture, the cell number of bFGF-PLGA group was statistically more than that of bFGF group ($P < 0.05$, Table 2).

Effect of bFGF-PLGA microspheres on the vitality of Schwann cells

The *A* values of cultured Schwann cells at different time points showed: 1, 2 days after plate culture, the *A* values of bFGF group were statistically higher than those of bFGF-PLGA group ($P < 0.05$); 3, 4 days after plate culture, the *A* values of bFGF group and bFGF-PLGA group showed no significant difference (P

> 0.05); 6, 8 days after plate culture, the *A* values of bFGF-PLGA group were statistically higher than those of bFGF group ($P < 0.05$, Table 3).

Effect of bFGF-PLGA microspheres on the mitotic cycle of Schwann cells

The examinations of mitotic cycles of Schwann cells at different time points showed that: 2 days after plate culture, the G2/M + S percentage of bFGF group was statistically higher than that of bFGF-PLGA group ($P < 0.05$); 4, 8 days after plate culture, the G2/M + S percentage of bFGF-PLGA group was statistically higher than that of bFGF group ($P < 0.05$, Table 4).

Table 2. Effect of bFGF-PLGA microspheres on the cytometry of Schwann cells ($\times 10^4/ml, \bar{x} \pm s$)

Groups	Time points (d)					
	1	2	3	4	6	8
bFGF	3.75 ± 0.43 *	9.00 ± 1.52 *	12.25 ± 2.18	16.42 ± 2.75	18.17 ± 2.13	20.08 ± 2.47
bFGF-PLGA	2.42 ± 0.38	5.67 ± 1.28	13.42 ± 1.89	16.83 ± 2.24	23.58 ± 2.67 *	25.33 ± 2.24 *
<i>t</i> values	4	2.902	0.7	0.203	3.610	3.261
<i>P</i> values	0.016	0.044	0.523	0.849	0.015	0.022

* Statistical difference between the bFGF group and bFGF-PLGA group, $P < 0.05$

Table 3. Effect of bFGF-PLGA microspheres on the viability of Schwann cells (*A* value, $\bar{x} \pm s$)

Groups	Time points (d)					
	1	2	3	4	6	8
bFGF	0.167 ± 0.023 *	0.209 ± 0.036 *	0.244 ± 0.026	0.272 ± 0.029	0.293 ± 0.030	0.321 ± 0.026
bFGF-PLGA	0.121 ± 0.011	0.136 ± 0.021	0.240 ± 0.040	0.275 ± 0.035	0.354 ± 0.031 *	0.387 ± 0.031 *
<i>t</i> values	3.179	3.039	0.158	0.115	2.935	2.865
<i>P</i> values	0.034	0.038	0.882	0.914	0.032	0.046

* Statistical difference between the bFGF group and bFGF-PLGA group, $P < 0.05$.

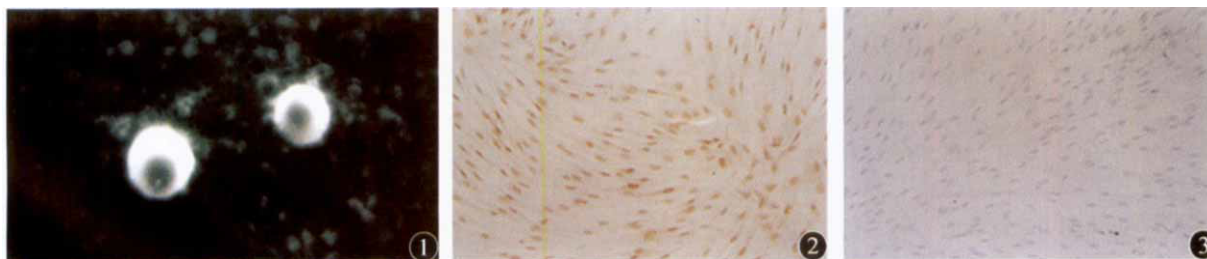


Fig. 1. SEM of the bFGF-PLGA microspheres; The morphology and the particle size of the microspheres are even and good ($\times 4000$)

Fig. 2. Immunohistochemistry staining of the Schwann cells showing the diffuse positive reaction in the cytoplasm (S100 IHC $\times 100$).

Fig. 3. Immunohistochemistry staining of the Schwann cells showing the negative reaction in the cytoplasm if the antibody is replaced by PBS (S100 IHC $\times 100$).

Table 4. Effect of bFGF-PLGA microspheres on the mitotic cycle of Schwann cells (G2/M + S percentage, $\bar{x} \pm s$)

Groups	Time points (d)		
	2	4	8
bFGF	19.70 \pm 3.80 *	13.17 \pm 3.53	9.83 \pm 2.41
bFGF-PLGA	11.47 \pm 2.51	19.03 \pm 2.45 *	18.80 \pm 2.07 *
<i>t</i> values	3.130	2.821	4.893
<i>P</i> values	0.035	0.037	0.008

* Statistical difference between the bFGF group and bFGF-PLGA group, $P < 0.05$.

DISCUSSION

By using natural or synthetic high molecule materials, drugs could be microcapsulated into microspheres with a diameter of 1-5 000 μm (commonly 2-250 μm) which own prolonged therapeutic effects, improved stability, reduced peculiar smell and side effects. The ideal microspheres prepared with biodegradable high molecular materials can achieve a stable characteristic and a good biocompatibility. After applying a single dose, the drug could keep releasing for a longer time. Furthermore, once complete release is completed, the coat material could self-eliminate *in vivo* through biodegradation. This can provide great facility for the use of steroidal drugs that have low dissolubility and slow velocity of infiltration, and polypeptide hormone drugs that would easily loose their bioactivities.^{3,4} As a polypeptide growth factor in possession of extensive bioactivities, bFGF is sensitive to heat or acid and has a short half-life time just like most growth factors. So this study utilizes the degradable material for the preparation of bFGF microspheres in order to achieve the controlled release of the drug and the maintenance of the stable local concentration in a certain period.

The carrier used in the preparation of bFGF controlled release microspheres could be divided into natural, semi-synthetic and synthetic materials, and the PLGA used in this study belongs to synthetic high molecule material, which hold the advantages of non-toxic, good biocompatibility, controllable degrading time and that the material and degradable production is harmless to the drug. Pean et al⁵ injected the PLGA microspheres into the cerebra of rats, and the result showed a normal morphology of the surrounding nerve cells and no toxic reaction was revealed. Lohmann et al⁶ planted the chondrocytes onto the PLGA frame

which had been preprocessed with PDGF, and found that not only the chondrocytes could adhere onto the frame, but also there were newly formed cartilages at the sites of plant. The PLGA material has been used in the preparation of the microspheres of several factors such as TGF- β , IGF, GH, and so on, and the controlled release effect and the conservation of drug's bioactivities are satisfactory. This study uses PLGA as coating material in the preparation of bFGF microspheres, and the results showed that the surfaces of the bFGF-PLGA microspheres were round and smooth, the spheres were symmetrical and no adhesion was found. The mean partical size was $1.552 \pm 0.015 \mu\text{m}$ and the mean size distribution was 1.310 ± 0.010 , which showed that the particle distribution was narrow and their sizes were uniform.

Drug loading and enveloping rates are two important indices used in the quality assessment of microspheric drug loading systems. The technique of preparation is an important one in the factors affecting the drug loading and enveloping rates. The main advantage of the multi-emulsion encapsulating mechanism is that when the dissolvent volatilizes, the polymers are splinted between the two aqueous phases and prevent the infiltration of drugs that are kept in the inner phase. So the microspheres prepared in this way have higher drug loading and enveloping rates compared with other methods using the same material.⁷ Blanco-Prieto et al⁸ used this method in preparation of somatostatin microspheres and the enveloping rate achieved 95%. In this study, the bFGF-PLGA microspheres prepared in the way of multi-emulsion encapsulating mechanism achieved a drug loading and enveloping rates of $(27.18 \times 10^{-3}) \pm (0.51 \times 10^{-3})\%$ and $(66.43 \pm 1.24)\%$ respectively, which have approved its efficiency and feasibility.

Another important index used in the assessment of drug loading system is the *in vitro* drug releasing rate. In this study, bFGF was released from microspheres constantly and stably within the 11 days period. The concentration of bFGF lies in a range of 71.47-89.81 ng/ml while the overall releasing rate was 72.47% at the 11st day, suggesting that in a short period after the preparation of bFGF-PLGA microspheres, bFGF could be released slowly and steadily.

The conservation of the bioactivities is the fourth

important index in a quality assessment of the microspheres after the preparation. Edelman et al⁹ prepared the bFGF-EVAc controlled release microspheres using the phase segregation method and 99% of the bioactivities of bFGF lost after the preparation. The efficiency of the microspheres is rather poor. The use of PLGA, which is degradable as the carrier for microspheres, not only could keep the release of the drug in a controlled way, but also protect the growth factor's activities in the facilities of the hydrophobic properties. In this study, the characteristic that bFGF could promote the proliferation of the Schwann cells was utilized.^{10,11} The conservation of the bioactivities of bFGF in the microspheres was assessed through the cytometry, cytoactivity detection and mitotic cycle analysis of Schwann cells. The result showed that at the beginning of the cultivation, the proliferation-promoting effect of the bFGF group preceded that of the bFGF-PLGA group; however, late in the cultivation, the proliferation-promoting effect of the bFGF-PLGA group preceded that of the bFGF group, which suggests that: 1) because of the equally total amount of bFGF, there is more amount of free bFGF in the bFGF group than the bFGF-PLGA group at the beginning of the cultivation, so there is more proliferation-promoting effect in the bFGF group; 2) late in the cultivation, most free bFGF in the bFGF group has lost their bioactivities while the bFGF that possesses their bioactivities keeps releasing in the bFGF-PLGA group and achieves a strong effect of promoting proliferation; 3) PLGA not only meets the requirement of controlled release, but also conserves the bioactivities of the released bFGF.

In conclusion, PLGA is an ideal carrier material in preparation of the bFGF microspheres, and bFGF-PLGA microspheres have a simple preparation technique and an excellent controlled releasing property. It could keep releasing bFGF possessing good bioactivities and thereupon stimulate the mitosis and proliferation of Schwann cells in a relatively prolonged period.

REFERENCES

1. Okada-Ban M, Thiery JP, Jouanneau J. Fibroblast growth factor-2. *Int J Biochem Cell Biol* 2000; 32(3): 263-267.
2. Klimaschewski L, Nindl W, Feurle J, et al. Basic fibroblast growth factor isoforms promote axonal elongation and branching of adult sensory neurons in vitro. *Neuroscience* 2004; 126(2): 347-353.
3. Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. *Int J Pharm* 2004; 282(1): 1-18.
4. Li XW, Xiao J, Deng XM, et al. Preparation of biodegradable polymer microspheres encapsulating protein with micron sizes. *J Appl Polym Sci* 1997; 66(4): 583-590.
5. Pean JM, Menei P, Morel O, et al. Intraseptal implantation of NGF-releasing microspheres promote the survival of axotomized cholinergic neurons. *Biomaterials* 2000; 21(20): 2097-2101.
6. Lohmann CH, Schwartz Z, Niederauer GG, et al. Pretreatment with platelet derived growth factor-BB modulates the ability of costochondral resting zone chondrocytes incorporated into PLA/PGA scaffolds to form new cartilage in vivo. *Biomaterial* 2000; 21(1): 49-61.
7. Choi SH, Kwon JH, Kim CW. Microencapsulation of insulin microcrystals. *Biosci Biotechnol Biochem* 2004; 68(3): 749-752.
8. Blanco-Prieto MJ, Besseghir K, Zerbe O, et al. In vitro and in vivo evaluation of a somatostatin analogue released from PLGA microspheres. *J Control Release* 2000; 67(1): 19-28.
9. Edelman ER, Mathiowitz E, Langer R, et al. Controlled and modulated release of basic fibroblast growth factor. *Biomaterials* 1991; 12(7): 619-626.
10. Timmer M, Robben S, Muller-Ostermeyer F, et al. Axonal regeneration across long gaps in silicone chambers filled with Schwann cells overexpressing high molecular weight FGF-2. *Cell Transplant* 2003; 12(3): 265-277.
11. Jungnickel J, Gransalke K, Timmer M et al. Fibroblast growth factor receptor 3 signaling regulates injury-related effects in the peripheral nervous system. *Mol Cell Neurosci* 2004; 25(1): 21-29.

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