Letter to the Editor

Controlling the Release of bFGF from Silk Fibroin Membrane*

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Since neurotrophic factor is easy to degrade and aggregate, it usually has a short half-life *in vitro*. To overcome this shortage, neurotrophic factor has been combined with the silk fibroin (SF) membrane to realize less degradation, optimal loading efficiency, sustained release, and good adsorption. By optimizing its binding conditions, main parameters were investigated and its optimal loading efficiency was obtained. bFGF was combined to SF membrane by layer by layer (LbL) static adsorption technique. The natural and nontoxic chondroitin sulfate (CS) was used as a crosslinking agent. Optimization was carried out in three aspects: the concentration of bFGF, the concentration of CS, and the reaction time. This experiment provides a better environment for the growth of cells and offers a new kind material of absorbing neurotrophic factor to meet increasing demand for biological materials.

Since silk fibroin (SF) can be used to prepared nerve conduits with a number of advantages including biocompatibility, chemical versatility, and controlled degradability, it is one of excellent natural biomaterials[1]. When long distance nerve defects is unable to be end-stitched to compensate neurological deficits, nerve graft would be the sole approach to repair the nerve deficits[2]. Various nerve conduits were prepared based on SF for peripheral nerve defect. It is urgent to find an ideal nerve graft to substitute the autologous nerve graft due to limited transplant autologous nerve sources, mismatch of organizational structure and size and the long denervation of transplant donor site. The ideal artificial nerve graft should include tissue engineering scaffold material, neurotrophic factors and seed cells. Scaffolds should have good mechanical properties, permeability, biocompatibility, and biodegradability, and neurotrophic factors should have the appropriate concentration and a suitable release rate. Therefore, it can be speculated that by containing a certain concentration of growth factor, the artificial nerve graft would be able to improve neurological defect repairing effect, especially for long-distance large nerve defects.

However, the growth factor usually has a short half-life, since it is easy to degrade and aggregate. Therefore, we have used layer by layer method to combine the factor bFGF to the SF membrane by a natural and nontoxic crosslinking agent chondroitin sulfate (CS). Layer by layer assembly is a powerful new film preparation technique that can be applied to the design of versatile biomaterials[3]. Through the optimization of neurotrophic factor binding conditions, the concentration of bFGF, the concentration of CS, reaction time and main parameters were investigated and the optimal loading efficiency of neurotrophic factor was obtained. And the enzyme-linked immunosorbent assay (ELISA) was used to measure and evaluate the sustained release of bFGF.

Raw silk fibers (from B. *mori* cocoons) were bought from Xinyuan Sericulture Company (Haian, China). The sericin coating of silk fibers was removed via degumming process of boiling in aqueous Na_2CO_3 (Sino harm Chemical Reagent Co., Ltd) solution as previously described[4]. Chondroitin sulfate exists in animal cartilage; the commodity is extracted from animal tissue preparation of acid mucopolysaccharide. After drying the silk fibroin membrane in the plate, bFGF (Millipore company) was combined with membrane through

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layer-by-layer (LbL) method[5]. Firstly, the silk membrane was immersed in the 0.3% acetic acid, and then coated with chondroitin sulfate (Amresco Company). After having been combined with CS, the membrane was washed with 0.1 mol/L NaCl (Sino harm Chemical Reagent Co., Ltd) solution (pH 5.6) three times, and dried in the room temperature. It was submerged in the bFGF solution to afford the bFGF combined SF membrane. The SF membrane was situated in the environment of saturated sodium sulfate steam for one month to make the membrane undissolved in water or something hydrophilic liquid[6]. Then, the membrane was disinfected with 70% alcohol and washed with phosphate buffered saline (PBS, 0.1 mol/L, pH 7.4) prior to use.

To get optimal loading efficiency of bFGF, the following main parameters were investigated. According to other researches[7], the concentration of original loading bFGF was 1 μg/mL, 5 μg/mL, 10 μg/mL, 15 μg/mL, 20 μg/mL, and 25 μg/mL. In the condition of one parameter being confirmed, the series concentration of chondroitin sulfate (CS) was 0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2 mg/mL, and 2.5 mg/mL, when the conjugation time was used as the last parameter, it was tested for 5 min, 10 min, 15 min, 30 min, 45 min, and 60 min. The amount of bFGF released from the fibroin film was analyzed by ELISA assay. The fibroin film was incubated in 0.5 mL PBS at room temperature. At each time interval, the PBS was collected and then the total was replaced with fresh PBS. Each experiment was repeated for three times.

PC12 cells were cultured on a 24-well culture dish at the density of 3×10^4 cells/well in Ham's F-12 Kaighn's modification (F12K) complete supplemented with 2.5% FBS, 15% horse serum, 100 U/mL penicillin, 100 μg/mL streptomycin. PC12 cells were cultured in a humid 5% CO_2 and 37 °C atmosphere. Mouse L929 cells, obtained from the Chinese Academy of Sciences (Shanghai Institute of Cell Biology), were seeded on a 60 mm culture dish at the density of 2×10^5 cells/well for incubation in RPM1640 medium (Gibco) supplemented with 10% FBS and antibiotics (Gibco) in a humid 5% CO_2 atmosphere. Each experiment was repeated for three times.

The control releasing bFGF from silk fibroin film system was established by the layer by layer method. Firstly, the undissolved SF membrane was immersed in 3% acetic acid to make the amino get positive charge. Next, the molecular CS charged with negative charge could be absorbed to the amino of silk fibroin. Thirdly, bFGF with positive charge could be absorbed to the CS. The silk fibroin solution was casted on the corning 24-well plate and the scanning electron microscope (SEM) image of the membrane. The surface of membrane was not so flat and dense, which benefited cells to be attached. After adsorption of bFGF, the surface of membrane did not change significantly, because the molecule of bFGF and chondroitin sulfate was nanoscale and surface morphology structure of the fibroin membrane was at a micron level.

Layer by layer assembly is sequential adsorption of oppositely charged macromolecular species, it is a powerful new film preparation technique that can be applied to the design of versatile biomaterials, with well-controlled interfacial, mechanical, and biological functions[8-9]. The SF films on the 24-well plate were prepared for cell culture. In the following experiments, 1 mL SF solution was placed on the six-well plate for convenient operation and experimental data processing. The computation formula of the binding bFGF was as follows: amount of binding bFGF=V1×C1-V2×C2 (V1=1 mL, V2 presents the volume after layer by layer experiment; C1 means the serials of bFGF concentrations, C2 is the concentration of bFGF after the layer by layer experiment tested by ELISA assay), which was applied for the optimization of layer by layer parameters. V2 might lose during the operation. Figure 1A shows one layer bFGF binding capacity of the membrane and demonstrates that the concentration of bFGF is under the 10 μg/mL, amount of one layer bFGF combined to the film presents uptrend. While the concentration of bFGF is 10 μg/mL or is greater than 10 μg/mL, the amount of binding does not rise. The concentration 10 μg/mL of bFGF is optimal threshold value. The concentration 10 μg/mL of bFGF is the optimal parameter, to get the best binding result, the other parameters also should be considered. Figure 1B shows the influence of serials concentration of chondroitin sulfate (CS) on the amount of binding one layer bFGF. We tested the influence of CS concentration on the bFGF binding efficiency by ELISA assay using the above formula. The result showed that bFGF was combined with silk fibroin membrane on the condition when chondroitin sulfate concentration was properly 1.5 mg/mL. Then, conjugation time of bFGF and CS was taken as variability. As shown by Figure 1C, the appropriate reaction time of CS and bFGF was 15 min; the maximum amount of bFGF binding to silk fibroin membrane was examined by ELISA assay.

Using the above conditions, the bFGF concentration was 10 μg/mL; the concentration of CS was 1.5 mg/mL; the combined time of bFGF and CS was 15 min, the cumulative amount of bFGF sustained releasing from silk fibroin membrane in 30 d was tested by ELISA assay. According to other researches on layer by layer method, the combined layers of bFGF and CS through attracting positive and negative charges were usually about ten layers[3,10]. After the preliminary experiment, ten combined layers of bFGF and CS were determined. As shown by Figure 1D, 4.5296 μg of bFGF was combined to silk fibroin membrane in 30 d. The releasing of bFGF sustained from 1 d to 16 d and the average daily releasing was 0.22 μg. Amount of bFGF from 17 d to 30 d released gradually reduced the average daily release by 0.025 μg; the release percentage of binding bFGF by this method was about 90%.

L929 cells were cultured on the SF membrane with bFGF, the membrane without bFGF, the membrane without bFGF but adding 1.5 mg/mL CS in the medium and the common cell culture plate. Furthermore, the cells were observed by optical microscopy after being cultured for 12 h. It could be seen that the number of L929 cells on the membrane combined with bFGF was more than on the culture plate and on the membrane without bFGF. Bright-field images indicate that all cells proliferate well and keep their normal configuration on all the substrates (Figure 2). The cells on the substrate combined with bFGF by layer by layer method extend their stretch extend and expand their spread. More cells on the culture plate present round character, indicating that the film has no cytotoxic effect under all used conditions but also can enhance the proliferation of the L929 cells.

The effect of bFGF slow releasing from SF membrane can not only sustain L929 cell growth, but also promote cell differentiation. The neurite outgrowth of PC12 cells was evaluated by optical microscope. Figure 3 shows the differentiation of PC12 cells cultured with the extract liquid of various substrates. It can be seen that neurite growth cultured in medium containing bFGF is significantly longer than other group without bFGF after 3 d.

10 μg/mL of bFGF solution, 1.5 mg/mL of CS solution and 15 min of bFGF and CS appropriate reaction time all created better condition, under which we examined the quantity of bFGF binding and releasing in one month. The average daily released bFGF was 0.15 μg; the release percent of binding bFGF by this method was 90%. Both of cultured PC12 cells and fibroblast cell line L929 kept alive in the membrane combined bFGF under optimized

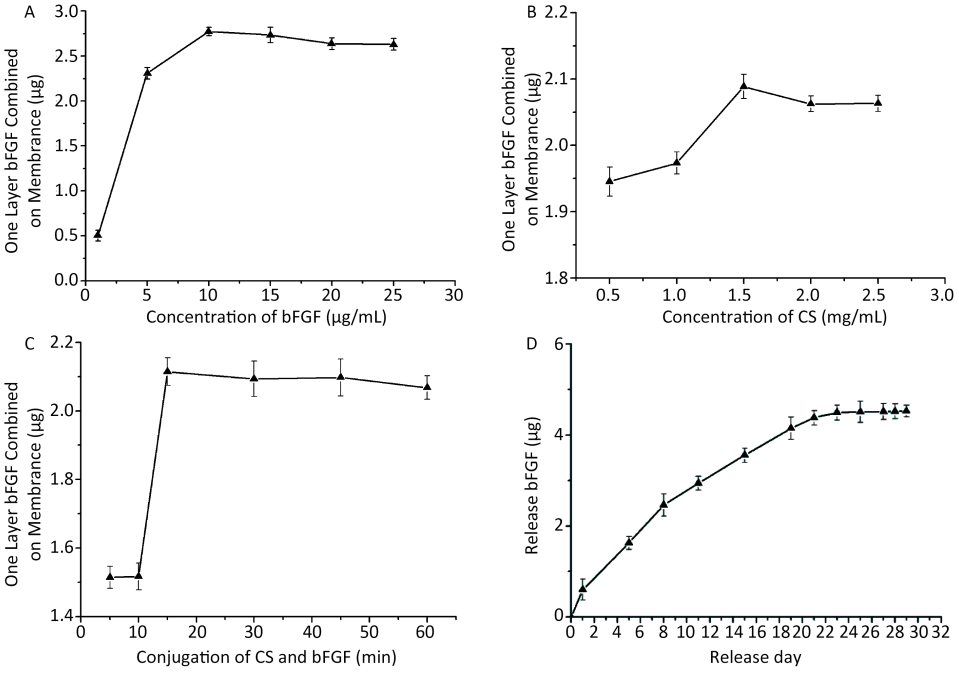

Figure 1. (A) bFGF binding capacity of the curve as bFGF as variable. (B) bFGF binding capacity of the curve as chondroitin sulfate (CS) as variable. (C) Conjugation time as variable release kinetics. (D) One month bFGF release profile.

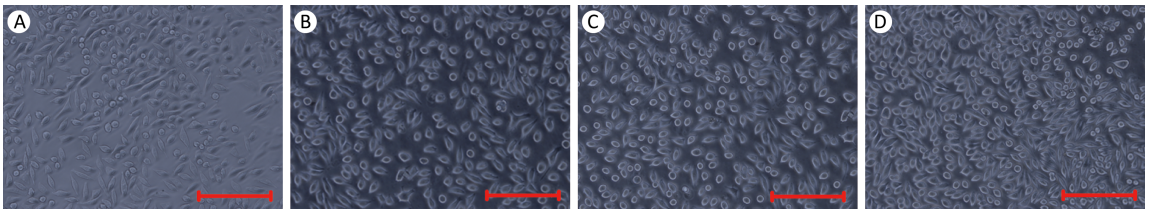

Figure 2. (A) L929 cells cultured in common dish. (B) L929 cells cultured on silk fibroin membrane without bFGF. (C) L929 cells cultured on silk fibroin membrane without bFGF by adding CS. (D) L929 cells cultured on silk fibroin membrane combined with bFGF. Bar=100 μm.

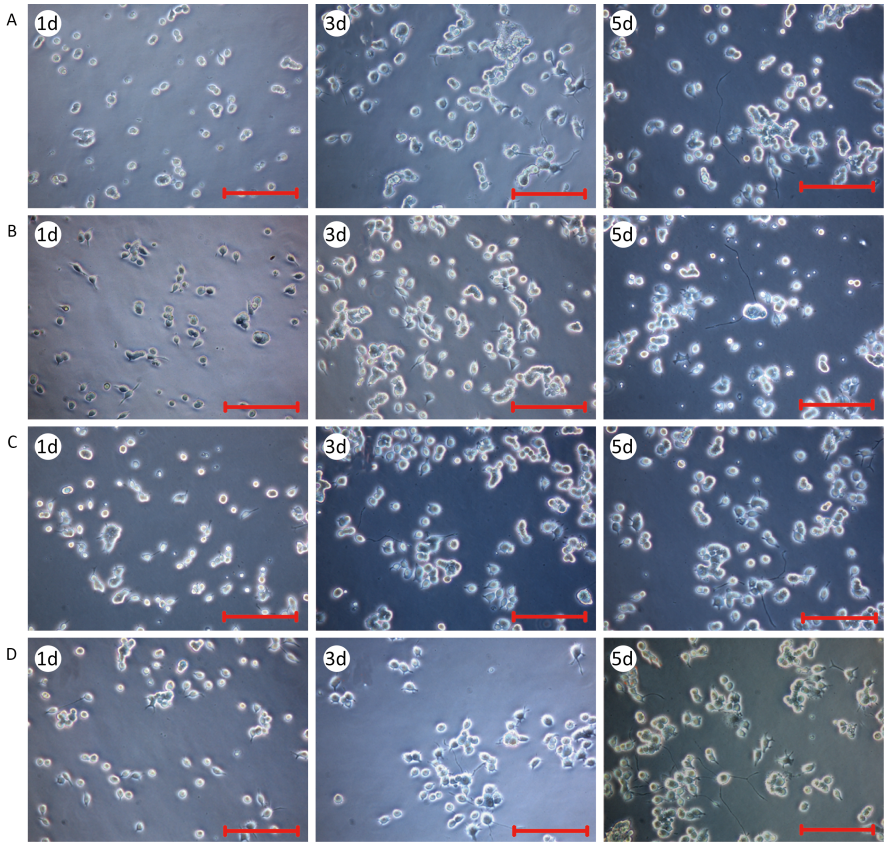

Figure 3. (A) PC12 cells cultured in medium without bFGF. (B) PC12 cells cultured in extract liquid from silk fibroin membrane. (C) PC12 cells cultured in the extract liquid from silk fibroin by adding 1.5 mg/mL CS. (D) PC12 cells cultured in the extract liquid from silk fibroin combined with bFGF. Bar=100 μm.

conditions. Silk fibroin is suitable for binding neurotrophic factors to promote the cells' proliferation and differentiation.

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