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Preparation and characterization of bFGF and BSA-loaded microspheres

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This article describes the preparation and characterization of BSA and bFGF-loaded PLGA microspheres and assesses the effect of entrapment procedure and in vitro *release conditions. In the experiments, changing the PVA concentration from 3 to 5% (w/v) in the pre-emulsion inner phase led to a change in particle size and encapsulation efficiency of BSA-loaded and bFGF-loaded PLGA microspheres from 1 to 0.7 µm and from 73 to 65%, respectively. The* in vitro *release of the bFGF from PLGA microspheres was much lower than BSA-loaded PLGA microspheres. For all of the formulations, BSA and bFGF retained integrity after the microencapsulation process as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Besides, the cell proliferation-stimulating activity of bFGF was evaluated by using BALB/c 3T3 cells and it was shown that the bioactivity of bFGF was increased by adding heparin to the release medium.*

Key words: PLGA – Microspheres – BSA – bFGF – SDS-PAGE.

Significant advances in biotechnology have resulted in the discovery of a large number of therapeutic and antigenic proteins [1]. For example, a number of growth factors (bFGF, PDGF, EGF etc.) have been identified as potential drug candidates for the treatment of various disorders. However, the *in vivo* application of biologically derived drugs remains limited due to problems in stability, short biological half-life and delivery [2]. Thus, the problem faced is now the development of suitable protein delivery devices [1].

It has been widely recognized that growth factors greatly contribute to tissue regeneration at different stages of cell proliferation and differentiation. However, successful tissue regeneration by the use of growth factors has not always been achieved. One of the reasons for this is the very short half-lives of growth factors in the body to sustain biological activities. Thus, it is necessary to sustain the required quantity of growth factors in order to enhance the *in vivo* efficacy [3, 4].

Basic fibroblast growth factor (bFGF or FGF-2) is a protein with a molecular mass of about 18 kDa, which was shown to exhibit proliferative activity in fibroblastic cells [5, 6]. It is an endogenous neurotrophin that is neuroprotective in cerebral ischemia following intracerebroventricular (i.c.v.) injection. bFGF was administered by i.c.v. injection because previous work had shown that it did not cross the blood-brain barrier (BBB) in pharmacologically significant amounts [7]. Thus, bFGF was shown to be neuroprotective following intravenous (i.v.) administration of high doses (135 μg/kg) in rats subjected to middle cerebral artery occlusion. However, clinical trials of bFGF in human subjects showed dose-associated side effects [7]. Therefore, systemic treatment should be avoided to prevent the potential undesirable effects [7, 8]. The discovery of high affinity binding of bFGF to heparin has accelerated its purification, characterization, and cloning [6]. Heparin increases the receptor affinity of bFGF, protects bFGF against thermal and pH-induced denaturation, and is essential for the mitogenic activity of bFGF stimulated cells [6, 9].

The objective of this study was to produce microencapsulated formulations of bFGF, in which the physical, chemical, and biological properties of the protein remain unchanged during encapsulation. It is particularly important, during these processes, to preserve the protein structure and thus bioactivity. Since bFGF is expensive, bovine serum albumin (BSA) was used as a model protein in the first part of this study to determine the influence of technological factors on the microsphere characteristics. PLGA was chosen as polymer for the preparation of microspheres since it is the most widely used and well characterized polymer for biodegradable microspheres. A model protein, BSA-loaded poly(D,L-lactide-co-glycolide) (PLGA) microspheres were prepared and characterized for optimization of formulation parameters that would be used for the actual formulation of the target protein bFGF. Stabilizers were also added to the formulation of bFGF-loaded PLGA microspheres to prevent the degradation of protein. In the second part of this study, bFGF-loaded PLGA microspheres were prepared and characterized.

I. MATERIALS AND METHODS

1. Materials

Human bFGF was obtained from Euromedex (France). Anti-rabbit IgG (whole molecule) peroxidase conjugate, anti-human fibroblast growth factor (anti-bFGF), BSA and dichloromethane, polyvinyl alcohol (M_w 72 000) were purchased from Sigma (USA) and 50:50 poly (D, L-lactide-co-glycolide) (Resomer RG 503H) from Boehringer Ingelheim (Germany). BCA Protein Assay Reagent Kit was provided by Pierce Biotechnology, Inc. (USA). Bio-Rad Mini Protean II Electrophoresis System was supplied by Bio-Rad (USA), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, calf serum and sodium pyruvate by Biological Industries (Israel) and penicillin G sodium-streptomycin sulfate solution by Biochrom (Germany). BALB/3T3 cell lines were purchased from the American Type Culture Collection (ATCC, USA). All other chemicals and reagents were of analytical grade.

2. Methods

2.1. Preparation of microspheres

2.1.1. Preparation of BSA-loaded PLGA microspheres

PLGA microspheres were prepared by a modification of the solvent extraction technique using a $w_1/o/w_2$ double emulsion system previously described [10, 11]. In brief, 3 ml PVA (3 or 5%, w/v) aqueous solution with 0.662% BSA was added to 18 ml PLGA organic solution (1%, w/v, in DCM). The mixture was emulsified by homogenization (Ultra-

Turrax model T25 (IKA Labortechnik, Germany) at 11,000 rpm in an ice bath for 2 min. Thereafter, this first emulsion was homogenized with a Silverson Model L4RT Laboratory Mixer (Silverson Machines Limited, UK) by dropping through a syringe needle into 150 ml of the PVA aqueous solution (0.4 or 3%, w/v) at 4,000 rpm in an ice bath for 3 h. The particles were centrifuged at 15,000 rpm for 25 min, washed three times in distilled water and freeze-dried. Preparation parameters are summarized in *Table I*.

2.1.2. Preparation of bFGF-loaded PLGA microspheres

bFGF-loaded PLGA microspheres were prepared using a previously described [10, 11] $w_1/o/w_2$ double emulsion system. Briefly, microspheres were prepared by emulsifying the inner aqueous phase (3 ml) containing 3 or 5% (w/v) PVA and 0.0027% (w/w) bFGF in dichloromethane (18 ml) containing 1% (w/v) PLGA with Ultra-Turrax model T25 (IKA Labortechnik, Germany) at 11,000 rpm in an ice bath for 2 min. Thereafter, this primary emulsion was dispersed through a syringe needle into 150 ml aqueous solution of 0.4 or 3% (w/v) PVA and homogenized with a Silverson Model L4RT Laboratory Mixer (Silverson Machines Limited, UK) at 4,000 rpm in an ice bath for 3 h. The particles were centrifuged at 15,000 rpm for 25 min, washed three times in distilled water and freeze-dried. Preparation parameters are summarized in *Table I*.

2.2. Characterization of microspheres

2.2.1. Particle size distribution

Particle size distribution of the PLGA microspheres was determined by laser diffractometry using a Malvern Mastersizer (Malvern Instruments Ltd., UK). Samples were prepared by suspending the microspheres in distilled water containing 0.01% (w/v) Tween 80 for 1 min in an ultrasonic bath. The average particle size was expressed as the volume mean diameter.

2.2.2. Surface morphology

The surface morphology of the PLGA microspheres was examined by scanning electron microscopy (SEM) (Jeol-SEM 1200 EX, Japan). Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a layer of gold at 150 Å thickness using a Bio-Rad apparatus.

2.2.3. Determination of protein loading

The loading of BSA and bFGF in the microspheres was determined by hydrolyzing triplicate samples of BSA-PLGA (10 mg each) and bFGF-PLGA microspheres (15 mg) in 250 μl of 2 M NaOH solution overnight. The samples were then neutralized with 250 μl of 2 M HCl. The BSA and bFGF contents in the samples were quantified by using the BCA protein assay [12].

2.2.4. *In vitro* release study

The *in vitro* protein release studies of the BSA and bFGF-loaded PLGA microspheres were carried out in PBS (pH 7.4). The freeze-dried microspheres (20 mg) were dispersed in 500 μl PBS and placed in a shaker bath at 50 rpm at 37 ± 0.5°C. The same release study was also carried out for bFGF in PBS containing 15% (w/v) BSA, 0.0025% (w/v) heparin, 0.01% (w/v) EDTA [13].

The BSA concentration in the supernatants was determined by the BCA protein assay.

The *in vitro* release of bFGF was quantified by using ELISA [13]. A 96-well plate was used during the assay. Samples or standards of bFGF in the release medium were added to each well and incubated overnight at 4°C. After washing, 1% BSA was added to the wells and the plate was kept at room temperature for 30 min. The wells were then washed, incubated with anti-bFGF overnight at 4°C, washed to remove any unbound protein, and then incubated with anti-rabbit IgG-horseradish peroxidase. Finally, substrate o-phenylenediamine with H_2O_2 was added for color development (30 min at room temperature). The reaction was stopped with 2.5 M H_2SO_4 and intensity of the color, which was proportional to the quantity of the bFGF bound, was measured by reading the absorbance at 490 nm. Each measurement was made in triplicate, and the bFGF level was determined from a standard curve generated for each set of samples assayed by the GraphPad Prism 4 Programme.

2.3. Protein integrity

The integrity of the encapsulated and released BSA and bFGF was determined by the SDS-PAGE [14]. BSA or bFGF-loaded PLGA microspheres (20 mg each) were incubated in 1 ml of cold acetone for 1 h at -20°C, centrifuged and the supernatant was then removed. The remaining BSA or bFGF pellets were lyophilized and reconstituted in PBS for analyzing the effect of the preparation process on the integrity of BSA. The released samples from the PLGA were also lyophilized and reconstituted in PBS. The BSA and bFGF samples, native BSA, native bFGF and a molecular weight reference marker (wide range, Sigma, USA) were loaded onto 12% acrylamide gel and run using the Bio-Rad Mini Protean II Electrophoresis System. The bands were visualized by Coomassie blue staining.

2.4. Cell culture

BALB/c 3T3 cells were maintained in DMEM supplemented with 10% new born calf serum, L-glutamine (0.584 g/l), sodium pyruvate (0.11 g/l), penicillin G sodium (50 U/ml) and streptomycin sulfate (50 μg/mL) at 37°C in a humidified incubator containing 5% CO_2. The cells were grown in 75 cm^2 culture flasks. The cells in exponential growth phase (confluent monolayer) were used in the mitogenic bioassay following trypsinization.

2.5. Bioactivity assay

In our studies, the bioactivity of bFGF was determined using a mitogenic assay. This assay is based on the dose-dependent induction of the cell proliferation by bFGF and the response was measured using the MTT stain.

bFGF standards (50 μl) diluted in the serum free medium or release samples were added to each well. Then, 50 μl of cell suspension (containing 5000 cells) was added to each well. The plate was incubated

Table I - Preparation parameters, encapsulation efficiencies and particle sizes of BSA-PLGA, bFGF-PLGA microspheres*.

Formulation code	Theoretical protein loading (%)	Concentration of PLGA (%)	Conc. of PVA in inner aqueous phase (%)	Mean diameter (μm)	Practical protein loading (%)	Encapsulation efficiency (%)
BSA-PLGA 3	0.662	1	3	1.087 ± 0.0012	0.483 ± 0.025	73.02 ± 0.02
BSA-PLGA 5	0.662	1	5	0.704 ± 0.0035	0.449 ± 0.011	67.65 ± 0.01
bFGF-PLGA 3	0.0027	1	3	1.261 ± 0.0012	0.0019± 0.057	72.20 ± 0.05
bFGF-PLGA 5	0.0027	1	5	0.714 ± 0.0032	0.0018 ± 0.025	65.47 ± 0.02

*See “Materials and methods” for the operating conditions.
BSA-PLGA 3: BSA-PLGA 3 formulation (3% PVA in inner aqueous phase); BSA-PLGA 5: BSA-PLGA 5 formulation (5% PVA in inner aqueous phase); bFGF-PLGA 3: bFGF-PLGA 3 formulation (3% PVA in inner aqueous phase); bFGF-PLGA 5: bFGF-PLGA 5 formulation (5% PVA in inner aqueous phase).