Journal of Applied Polymer Science

Preparation of Bioactive Glycosylated Glial Cell-Line Derived Neurotrophic Factor-Loaded Microspheres for Medical Applications

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ABSTRACT: Poly (lactic-*co*-glycolic acid) (PLGA)-coated gelatin microspheres containing glial cell-line derived neurotrophic factor (GDNF) were developed by thermal gelation through a water-in-oil emulsion technique. Gelatin types (A and B) at four different pH levels were investigated for their influences on the morphology, the microsphere size, the zeta potential, and the swelling ability. The encapsulation of GDNF and the release characteristics of GDNF were also determined using enzyme-linked immunosorbent assay (ELISA). The maximum cumulative released amounts of GDNF from the microspheres were increased from 50 to 90% after 4 d (based on the actual amount of the GDNF). Thus, the release of the GDNF contents in the microspheres depends on the amount of GDNF. Trigeminal ganglion cells (TGCs) were used to study the bioactivity of GDNF released from the microspheres, which was proven to retain its bioactivity in promoting the TGCs' neurite outgrowth. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40168.

KEYWORDS: drug delivery systems; biomedical applications; biomaterials; biocompatibility; biodegradable

Received 24 June 2013; accepted 7 November 2013
DOI: 10.1002/app.40168

INTRODUCTION

The degradation of proteins is one of the most significant limitations for their utilization, due to their short half-lives and susceptibility to enzymatic degradation and absorption.[1] Therefore, the protection of proteins to prolong their efficacy must be studied. The design of a sustained proteins delivery system has been developed in therapeutic proteins. Examples of carriers for the delivery of proteins are liposomes,[2] nanoparticles,[3] microspheres,[4] and emulsions.[5] Among these, microspheres are considered as an efficient method for protein delivery.

The controlled delivery of proteins via microspheres is advantageous because they can be administered by injection or ingestion. Furthermore, microspheres can maintain constant protein levels and biologically active conformations. Microspheres are generally fabricated from biopolymers and biodegradable polymers, mainly due to their complete biodegradation. Among various biopolymers, gelatin microspheres have been developed as carriers for the delivery of basic fibroblast growth factor (bFGF),[6,7] transforming growth factor (TGF),[8] and plasmid DNA.[9] On the other hand, Poly(lactic-*co*-glycolic acid) (PLGA) microspheres have been developed as carriers for the delivery of bFGF[10] and TGF-β_1.[11] While gelatin was chosen mainly for its ease of fabrication into microspheres and its full biodegradability, PLGA was chosen for its ability to prolong the release of the encapsulated entity.

Gelatin is a biopolymer, derived from denaturation of collagen.[12] Two common variants of gelatin are types A and B, depending on the hydrolytic method with which it is obtained. A drastic change to the amide into the carboxyl groups of collagen occurs more readily in the alkaline process (type B gelatin), while the acidic process causes only a slight change to the amide groups (type A gelatin). As a result, some physic-chemical characteristics of the two types of gelatin are different. One important characteristic is the isoelectric point (IEP) of the two types of gelatin. While type A gelatin shows practically no change in the IEP value, while type B gelatin shows a lower IEP value, when comparing with the collagen.[13] As a result, the different IEP values of gelatin lead to a complex of electrostatic interactions between oppositely charged molecules.[13] Due to its natural origin, gelatin has been widely used in food,[14] tissue engineering,[15] gene therapy,[16] and drug delivery.[17]

Hariraksapitak et al.[18] reported the preparation of gelatin microspheres containing crude bone protein (CBP) and the

impregnation of these CBP-incorporated gelatin microspheres in porous composite hyaluronan (HA)-gelatin scaffolds. The release characteristics of CBP from the gelatin microspheres and, hence, the scaffolds were studied. The study showed that the release of CBP from the microspheres, in comparison with that from the scaffolds, was too fast, and there was only a short period of a sustained release. To prolong the release of protein molecules from gelatin microspheres, another polymer, which exhibits a slower releasing rate of an encapsulated substance when being fabricated into microspheres, can be used to modify the gelatin microspheres.

PLGA is a class of synthetic biodegradable polyesters of lactic and glycolic acids. By varying the molar ratios between the two constituents, PLGA of diverse properties can be obtained. PLGA has various advantages, such as improved a long-term drug release rate and ease of fabrication, not to mention its inherent properties like its excellent biocompatibility and biodegradability.[19–21] Because of these advantages, PLGA is used in many medical and pharmaceutical applications, such as tissue engineering and controlled drug or protein delivery.[22–25]

Glial cell-line derived neurotrophic factor (GDNF) is expressed in an adult brain and can be isolated from murine B49 glial cell line.[26] GDNF is a protein, the structure of which contains a disulfide-linked homodimer composed of amino acids in the sequence. GDNF has been regarded as one of the most effective growth factors that promote the survival of mesencephalic dopamine neurons in culture, and is one the most common factors that support the survival of motorneurons.[27–29] Clavreul et al.[30] investigated the effect of GDNF on 6-hydroxydopamine (6-OHDA)-treated dopaminergic neurons *in vitro*, and the results were ascribed to the neuroprotective effect of GDNF. Tan et al.[31] developed PLGA/gelatin microspheres as carriers for the delivery of an active substance to support the attachment, the proliferation, the viability and the secretion of glycosaminoglycans (GAGs) of chondrocytes.

The aim of this study was to prepare PLGA-coated gelatin microspheres that had been loaded with GDNF by an emulsion technique. The effects of two types of gelatins at different pH's on morphology, size, zeta potential value, and swelling behavior of the GDNF-loaded microspheres, as well as the release characteristics of GDNF from the microspheres in a bovine serum albumin (BSA)-containing phosphate buffer saline solution were investigated.

MATERIALS AND METHODS

Materials

Gelatin from porcine skin (type A, Bloom no.170–180) was purchased from Fluka (Switzerland), while that from bovine skin (type B, Bloom no.175–225) was purchased from Sigma-Aldrich (USA). PLGA ($M_w \approx$ 5000–15,000 Da) with a weight ratio between lactic acid and glycolic acid at 50 : 50 was purchased from Sigma-Aldrich (USA). Recombinant rat GDNF was purchased from R&D Systems (USA) (Cat. No.: 512-GF-010). Human GDNF MAb (monoclonal rat IgG1 Clone #27106; Cat. No.: MAB212) and human/rat GDNF affinity purified polyclonal Ab (Cat. No.: AF-212-NA) were also purchased from R&D Systems (USA). 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and the stop reagent for the TMB substrate were purchased from Thermo Scientific (USA). Glutaraldehyde (GTA; 5.6*M* aq. solution) was purchased from Fluka (Switzerland). Acetone (AR grade) and chloroform (AR grade) were purchased from Lab-Scan (Asia) (Thailand). All chemical agents were of analytical grade and used without further purification.

Preparation of PLGA-Coated Gelatin Microspheres Containing GDNF

PLGA-coated gelatin microspheres containing GDNF were created using a thermal gelation technique,[18] with slight modifications. Type A or B gelatin powder was first dissolved in distilled water at 40°C to prepare a stock solution of gelatin at 15% (w/v). To study the effect of pH, various pH levels (i.e., 3, 5.2, 7.4, and 10) were adjusted by using either 1*N* HCl or 1*M* NaOH. GDNF at a concentration ranging between 0.625 and 10 ng/mL was then added under constant stirring for 10 min to the solution. Next, a PLGA solution, i.e., 120 mg in 2 mL of dichlorometane : acetone (3 : 1 v/v), was added into the gelatin/GDNF solution under constant stirring for 10 min. The mixture was then added drop-wise into 200 mL of soya bean oil under constant agitation by means of a homogenizer operating at 1000 rpm at 40°C for 10 min to finally obtain an water-in-oil emulsion. The temperature of the emulsion was then cooled down to 4°C in an ice-water bath. After 30 min of constant stirring, 200 mL of acetone at 4°C was added and the mixture was stirred for 60 more minutes to dehydrate the microspheres. The microspheres were filtered, washed with pre-cooled acetone to remove residual oil, filtered again and finally freeze-dried at room temperature for 24 h.

The microspheres were cross-linked in 10 mL of an acetone/water mixture (2 : 1 v/v) that contained 1% (w/v) GTA solution. The mixture was stirred at 4°C and 500 rpm for another 1 h. The cross-linked microspheres were washed with 200 mL of pre-cooled acetone and collected by filtration under a vacuum. The cross-linked microspheres were then placed in 20 mL of 10 m*M* glycine aqueous solution, containing 0.1 wt % of Tween 80 and subsequently shaken at 37°C at 50 rpm for 1 h to block the residual aldehyde groups of the unreacted GTA. The cross-linked microspheres were again washed with pre-cooled acetone, filtered under a vacuum, and finally freeze-dried at room temperature for 24 h. The microspheres were stored at 4°C until of further use.

Characterization of the Microspheres

Morphological Observation. Morphologies of the neat and the GDNF-loaded types A and B gelatin microspheres, that had been prepared at various pH levels, were studied with a JEOL JSMe-5200 scanning electron microscope (SEM), operating at an accelerating voltage of 15 kV. Before SEM observation, each of the specimens had been mounted onto a carbon conductive tape on a copper stub and vacuum-coated with gold using a JEOL JFC-1100 sputtering device.

Size of Microspheres. Size of the microspheres was evaluated by a polarizing optical microscope (DMRXP, Leica) at 20× magnification. The optical images were analyzed with a UTHSCSA Image Tool software (version 3.0), to determine the

diametric information of the microspheres. For each sample type, atleast 100 microspheres from various optical images were analyzed. The data were reported as average values.

Swelling of the Microspheres. Swelling behavior of the cross-linked types A and B gelatin microspheres that had been prepared at different pH levels was investigated after incubation at 37°C in 10 mL of 10 m*M* phosphate buffered saline (PBS) in the presence of 0.15*M* NaCl. After 24 h, the size of the swollen microspheres was analyzed according to the previously described procedure in the previous sub-section. For each sample type, at least 100 microspheres were analyzed and their swelling behavior was quantified based on the following eq.:

$$\text{Swelling ratio} = D_{\text{swell}} \times 100 / D_{\text{dry}} \quad (1)$$

where D_{swell} and D_{dry} represent the size of the microspheres after and before the incubation.

Zeta Potential. Electrostatic mobility in terms of the zeta potential of the microspheres was analyzed using an electrophoretic analyzer (3.0+, Zeta-Meter). Briefly, a suspension of microspheres in deionized water was placed in an electrophoresis cell. The electrodes were inserted into the cell and connected to the Zeta-Meter 3.0+ unit. The microspheres moved towards either of the electrodes when the electrodes were energized. If they moved to the left, they were negatively charged, and if they moved to the right, they were positively charged. Individual microspheres were recorded under a microscope when they moved across the grid lines. The zeta potential values were measured when the microspheres moved to a designated point. At least 10 individual microspheres were studied for each sample, and the data were averaged and reported.

Actual GDNF Content

The actual amount of GDNF in the microspheres was quantified using the enzyme-linked immunosorbent assay (ELISA) method, as reported earlier.[32] Initially, 96-well microplates were coated with the diluted monoclonal anti-GDNF antibody in 0.025*M* carbonate with a pH of 8.2 at 4°C. After 24 h, the solution in the microplates was withdrawn for 1 h, and blocking solution was added to block the remaining free sites after microplate coating. Each sample was then dissolved in 1 mL dimethyl sulfoxide (DMSO) and the mixture was vortexed for 10 min. Then, 0.5 mL of the solution was pipetted into plates at room temperature. After 6 h, the microplates were washed at least five times with PBS, and the PBS was replaced with purified Mouse Monoclonal IgG1. The microplates were incubated at 4°C for 24 h. Afterwards, the microplates were washed five times with PBS, and the GDNF Affinity Purified Goat IgG1 was added to each well and incubated at room temperature for 2 h. The microplates were then washed five times with PBS. Later, the TMB substrate solution was added and incubated in a dark room at room temperature for 15 min. Finally, a stopping solution was added to stop the enzyme reaction. The concentration of the GDNF in the samples was further determined by a microplate reader at 450 nm and referenced to a GDNF standard curve. The experiment was completed in triplicate. The encapsulating efficiency of GDNF (EE) and the loading capacity (LC) of microspheres were calculated using the following equation:

$$\text{Encapsulating Efficiency (\%)} = \text{total ng GDNF encapsulated} \times 100 / \text{initial ng GDNF loaded} \quad (2)$$

and

$$\text{Loading capacity (\%)} = \text{total ng GDNF encapsulated} \times 100 / \text{total mg microspheres} \quad (3)$$

In Vitro GDNF Release

The release characteristics of the GDNF with microspheres were determined by measuring the amount of GDNF in the supernatant. Each of the samples (1 mg) was immersed in 0.5 mL of 10 m*M* PBS with 150 m*M* NaCl, which consisted of 0.1% BSA and sodium azide 0.02% (w/w). The samples were incubated in a shaking water bath at 37°C with constant shaking at 40 rpm. At each time point, 500 μL of the supernatant medium (i.e., the sample solution) was withdrawn and an equal volume of fresh medium was replenished to maintain a constant volume of the medium. The amounts of GDNF in the sample solutions were analyzed by ELISA. The experiment was assayed in triplicate, and the mean value and the standard deviation were calculated at every time point.

Cell Culture

Neural stem cells were used to study the cytotoxicity of the microspheres. These cells were isolated from the E11.5 mouse telencephalon. Isolated cells were cultured as a monolayer in minimum essential medium (Neurobasal Medium; Gibco) supplemented with 1% B-27 (Gibco, USA), 2 m*M* L-glutamine (Gibco, USA), Penicillin-Streptomycin (Gibco, USA) and 5 μg/mL of heparin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and passaged every 3 days.

Trigeminal ganglion cells (TGCs) were used to analyze the bioactivity of the GDNF released from the microspheres. TGCs were isolated from Wistar rats and cultured in DMEM/F12 medium (Hyclone, USA) supplemented with 1% B-27 (Gibco, USA), 0.6% glucose (Sigma, USA), 1 m*M* pyruvic acid (Gibco, USA), 2 m*M* L-glutamine (Gibco, USA), Penicillin-Streptomycin (Gibco, USA) and 50 μg/mL of insulin (Gibco, USA). TGCs were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Studies

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay for measuring the cell cytotoxicity of cells in culture. MTT is founded on the conversion of the cleavage of the yellow tetrazolium salt into purple formazan by metabolically active cells. The number of viable cells is proportional to the amount of purple formazan crystals formed. The cytotoxicity of microspheres was determined by the indirect cytotoxicity using neural stem cells as the reference cells. The microspheres were sterilized, and then the cells were plated at a cell density of 4×10^4 cells/well in 24-well culture plates at 37°C with 5% CO_2 in serum-containing MEM. Neural stem cells were incubated with the medium of non-loaded microspheres (control) and with the purified rat recombinant GDNF-loaded microspheres. After 24 h as the control condition, the medium was replaced with an extraction medium. After 24,

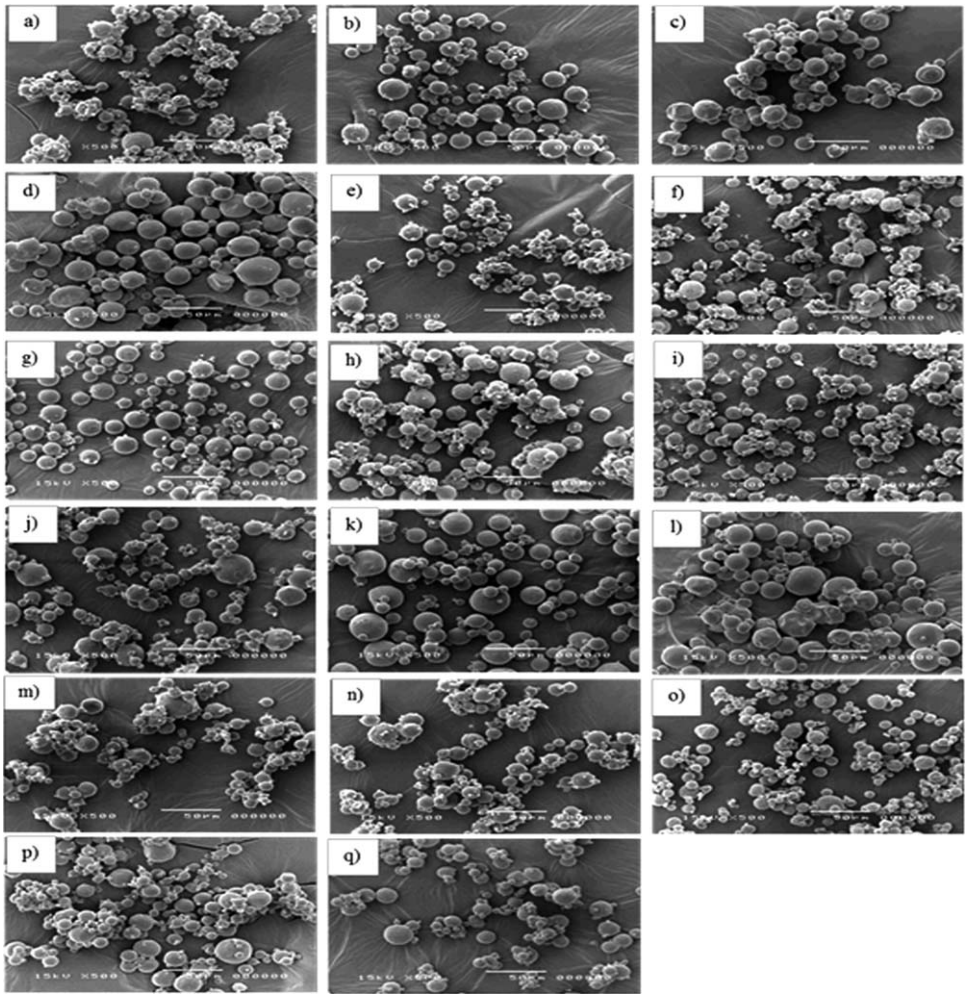

Figure 1. SEM images illustrating morphology of the gelatin microspheres both before and after cross-link with 100 m*M* GTA prepared with gelatin type A at pH 3.0 (a)–(e), pH 5.2 (b)–(f), pH 7.4 (c)–(g), pH 10.0 (d)–(h), and gelatin type B at pH 3.0 (f)–(m), pH 5.2 (j)–(n), pH 7.4 (k)–(o) and pH 10.0 (l)–(p) and PLGA-coated gelatin microspheres containing GDNF (q). The images present that the gelatin microspheres are spherical in shape with small pores present on a smooth surface and the surface of the GDNF-loaded gelatin microspheres was smooth.

48, and 72 h of cell culture in the extraction medium, each extraction medium was replaced with 500 μL/well of MTT solution (0.5 mg mL^{-1}). After 1 h of incubation at 37°C and 5% CO_2, each of the wells was filled with 1 mL/well of dimethylsulfoxide (DMSO) after removal of the MTT solution. Later, the absorbance of each well was reported on a Thermospectronic Genesis10 UV/Visible spectrophotometer at 540 nm. The experiment was performed in triplicate. The relative cell viability (%) was calculated using the following equation:

$$\text{Cell viability} = A_{\text{test}} \times 100 / A_{\text{control}} \quad (4)$$

where A_{test} is the absorbance of the samples and A_{control} is that of the control sample for 24 h.

In Vitro Bioactivity Studies

TGCs were plated onto a 24-well culture plate, incubated with the non-loaded microspheres (control) and with the 20 ng/mL of purified rat recombinant GDNF-loaded microspheres. After 5 days, TGCs were stained with beta-III tubulin and were captured with a Carl Zeiss Axio Observer Z1 Microscope. Next, the neurite outgrowth was analyzed with AxioVision software V.4.8 ($n = 50$).

Statistical Analysis

A one-way analysis of variance (ANOVA) and Scheffe's post hoc test in SPSS (SPSS, USA) were used to analyze the data, which are expressed as the mean ± standard deviations. The statistical significance for all cases was indicated at $P < 0.05$.

RESULTS AND DISCUSSION

Morphological Analysis

SEM was used to analyze the morphologies of the gelatin microspheres type A and B at different pH levels and the PLGA-coated gelatin microspheres containing GDNF. The pictures of the gelatin microspheres type A and B at different pH levels are exhibited in Figure 1(a–p). The images show that the microspheres are spherical in shape with small pores present on a smooth surface. Figure 1(q) illustrates the surface morphology of GDNF-loaded microspheres prepared with PLGA. The surface of the GDNF-loaded gelatin microspheres was smooth, with no pores on it.

Furthermore, both the gelatin microspheres and the PLGA-coated gelatin microspheres containing GDNF existed as large

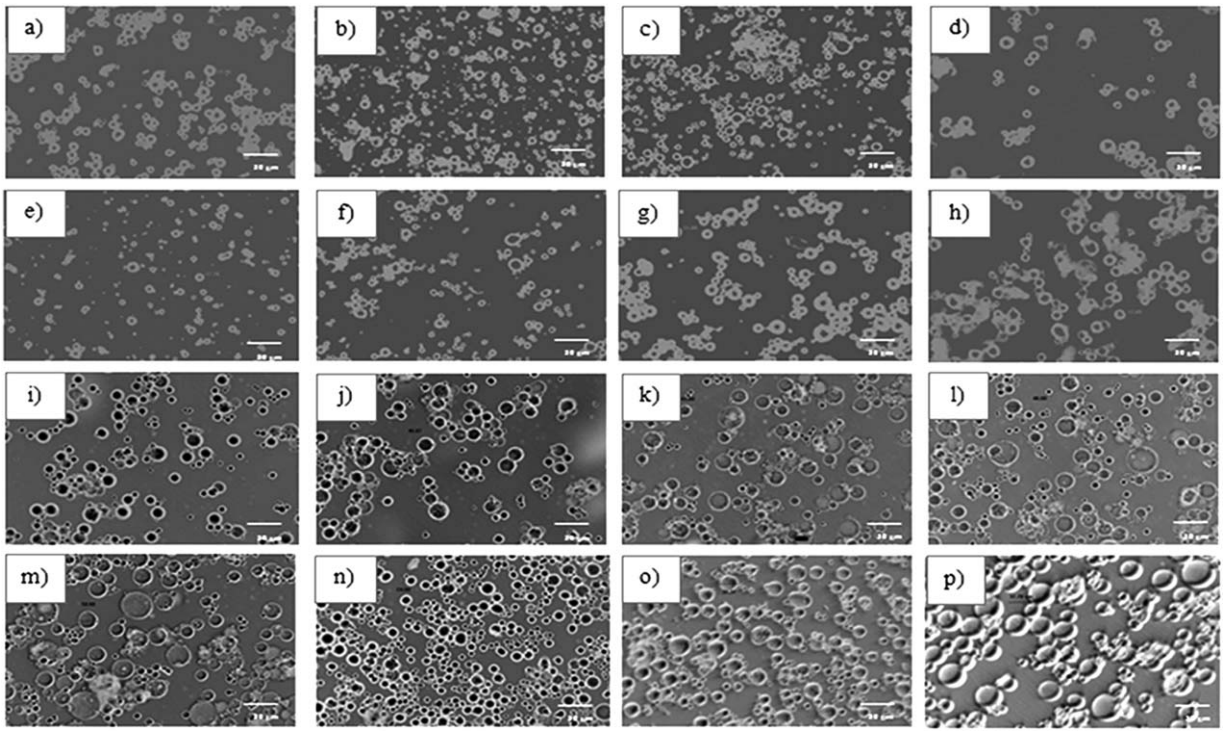

Figure 2. Optical images illustrating morphology of the uncrossing dry gelatin type A microspheres at pH 3.0 (a), pH 5.2 (b), pH 7.4 (c), pH 10.0 (d), gelatin type B microspheres at pH 3.0 (e), pH 5.2 (f), pH 7.4 (g), pH 10.0 (h), and cross-linked wet and swelling gelatin type A microspheres at pH 3.0 (f), pH 5.2 (i), pH 7.4 (j), pH 10.0 (k), and gelatin type B microspheres at pH 3.0 (m), pH 5.2 (n), pH 7.4 (o), pH 10.0 (p). The microspheres performed an ability to absorb water and increase in size after swelling in PBS were studied at 37°C for 24 h, as shown by the hydrated swelling behavior

aggregates due to the direct contact between the adjacent particles once the solvent was expelled during the preparation of the microspheres. The electrical charge on the surface of the particles was diminished in a dry environment, so that the electrostatic repulsive force was also weakened; as a consequence, the repulsion among particles was not exhibited.

Swelling Studies

The swelling ratio and the swelling behavior of the gelatin microspheres type A and B at different pH levels before and after swelling in PBS were studied at 37°C for 24 h to encapsulate the GDNF into the microspheres. The diameters of the microspheres from at least 100 freeze-dried and wet samples were measured and calculated, as reported in Figure 2 and Table I. The microspheres demonstrated an ability to absorb water and increase in size, as shown by the hydrated swelling behavior reported in Figure 2(i–p). The fold swelling ratios for microspheres were found to range from 1.1- to 2.0-fold. The pH difference did not influence the swelling ratio, regardless of the gelatin type. The result obtained in the present work corresponds to the findings of Vandervoort et al.[33]

Particle Size

The resulting size and the size distribution of the microspheres the uncross-linked microspheres ranged from 12 to 33 μm for gelatin A microspheres, while gelatin B in the uncross-linked, cross-linked, and swelling conditions are presented in Figure 3. The size of microspheres varied from 13 to 34 μm. Thus, the average size was not influenced by the type of gelatin. The effect of the pH level on the diameters of the microspheres was also studied. The results indicated that sizes of gelatin type A prepared at pH 3.0 and 10.0 differ in particle size. Microspheres prepared at pH 3 showed a diameter of 12 μm, compared with 33 μm for pH 10.0. The difference in microsphere size could be related to the protonation or deprotonation of the amino or carboxylic acid in the gelatin molecules.[33]

The cross-linking also changed the size of the microspheres. The particle sizes of the microspheres ranged from 11 to 32 μm, which is probably due to the difference in the cross-linking

Table I. Swelling Ratio of the Gelatin Microspheres Prepared With Gelatin Types A and B at Various pH levels

Type	Condition	Swelling ratio
Gelatin A	pH 3.0	1.60
	pH 5.2 (physiologic)	2.00
	pH 7.4	1.76
	pH 10.0	1.16
Gelatin B	pH 3.0	1.78
	pH 4.95 (physiologic)	1.37
	pH 7.4	1.76
	pH 10.0	1.62

The microspheres demonstrated an ability to absorb water and increase in size after swelling in PBS was studied at 37°C for 24 h. In each group, the data are significantly different at $P < 0.05$.

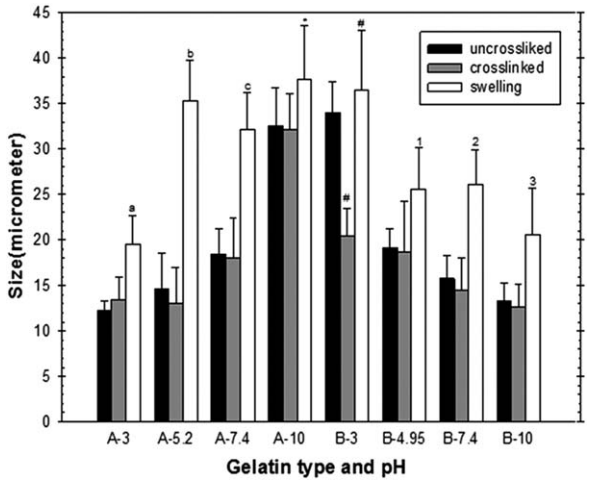

Figure 3. Particle sizes of the gelatin microspheres prepared with gelatin types A and B at various pH conditions by polarizing optical microscopy (DMRXP, Leica) at 20× magnification ($n = 100$). The average size was influenced by the pH level but the average size was not influenced by the type of gelatin. The size of microspheres varied from 11 to 34 μm. a,b,c, *, #, 1, 2, 3 are significantly different at $P < 0.05$; compared with the uncross-linked microspheres in each group.

in the gelatin: when the intensity of cross-linking was increased, there was a decrease in the particle size and a denser network.[34] However, the particle size of the swelling microspheres was much larger than those of the uncross-linked microspheres and the cross-linked microspheres, with an average size varying from 19 to 37 μm.

Zeta Potentials

The surface charge values of the gelatin microspheres are shown in Figure 4. The effect of the gelatin type and pH on the zeta potential values was investigated for all conditions. All zeta potential values of both gelatin A and gelatin B at different pH levels were obtained. At pH 10.0, the average zeta potential values were −60 and −45 for gelatin A and gelatin B, respectively, while gelatin type A and B were positive at pH 3.0 and 5.2. However, gelatin type A had a positive charge, but gelatin type B had a negative charge. Thus, both the type of gelatin and the pH level influence the zeta potential values of microspheres. This characteristic allows for electrostatic interactions between gelatin carriers with negatively charged and positively charged therapeutic agents (GDNF).

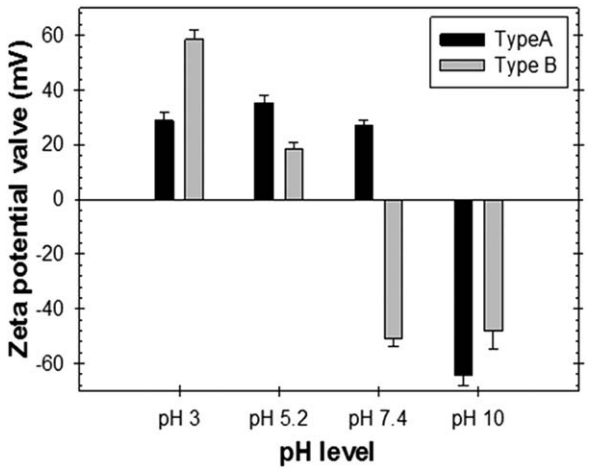

Figure 4. Zeta potential values of the gelatin microspheres prepared with gelatin types A and B at various pH ($n = 30$). The type of gelatin and the pH level influence the zeta potential values of microspheres. This characteristic allows for electrostatic interactions between gelatin carriers with negatively charged and positively charged therapeutic agents (GDNF). In each group, the data are significantly different at $P < 0.05$.

Loading of GDNF in Microspheres

The amount of GDNF loaded in the microspheres was evaluated by ELISA. The influence of the drug loading was determined as the percentages of the encapsulation efficiency of GDNF within the microspheres and the LC of the microspheres, as presented in Figure 5.

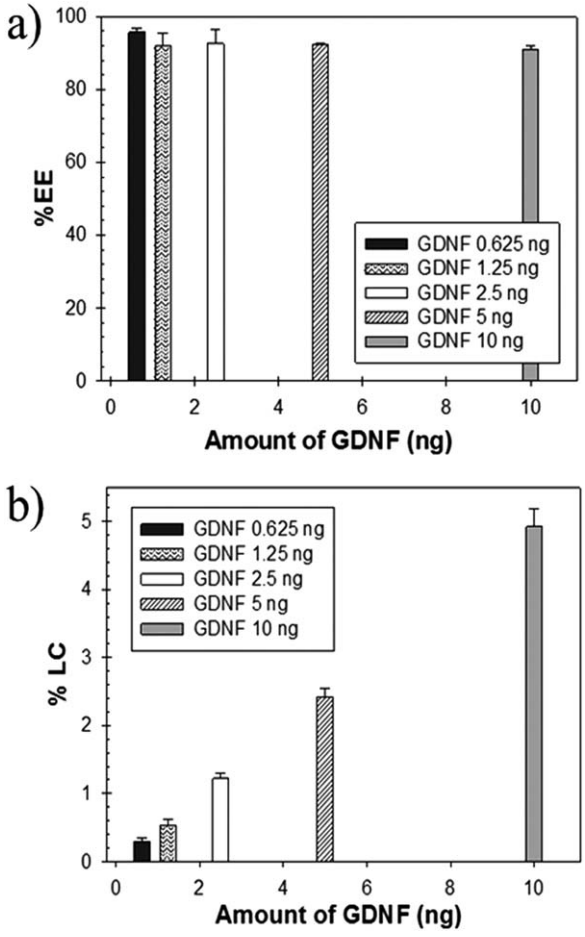

Figure 5. Encapsulating efficiency of GDNF within the PLGA-coated gelatin microspheres a) ($n = 3$) and LC of GDNF within the PLGA-coated gelatin microspheres b) ($n = 3$). This phenomenon could be described based on polyion complexation. The GDNF was positively charged, and PLGA and gelatin type A were negatively charged to sustain the release of protein drugs from polymer matrices and Figure 5b illustrates the increasing LC of the prepared microspheres might be increased with an increase in the concentration of GDNF. In each group, the data are significantly different at $P < 0.05$.

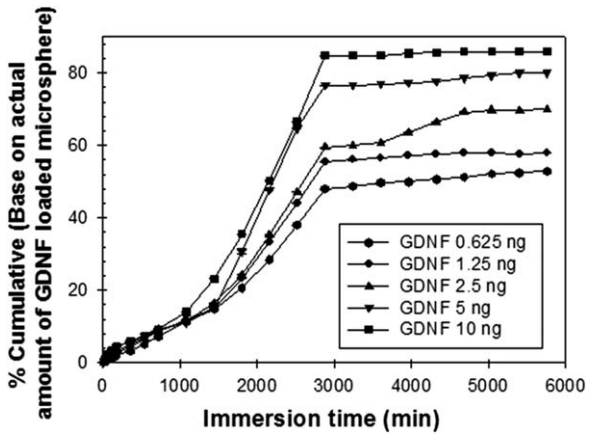

Figure 6. Cumulative release (%) of GDNF from the PLGA-coated gelatin microspheres in 0.15*M* NaCl–PBS containing of 0.1% BSA ($n = 3$). All *in vitro* release profiles demonstrated a biphasic modulation. The first phase was characterized by a relatively rapid initial release and followed by a second slower phase.

As observed, the encapsulation efficiency of GDNF ranged from 95 to 97% with an average of 96% for all samples at concentrations of GDNF from 0.625 ng /mL to 10 ng /mL, as shown in Figure 5(a). This phenomenon could be described based on polyion complexation.[35,36] Polyion complexes are formed by electrostatic interactions between positively charged and negatively charged species. The GDNF was positively charged, and PLGA and gelatin type A were negatively charged. It was expected that direct mixing of the GDNF in the gelatin aqueous solution containing PLGA solution would promote electrostatic interactions between those two oppositely charged molecules to sustain the release of protein drugs from polymer matrices.

The LC exhibited the capability of the microspheres to contain a therapeutic growth factor. The LC of GDNF varying from 0.5 to 5% was observed in Figure 5(b). The increasing LC of the prepared microspheres might be increased with an increase in the concentration of GDNF. Furthermore, this value was lower than the encapsulation efficiency of GDNF. In our opinion, it was caused because the initial amount of the GDNF was much smaller than the weight of microspheres during the preparation of the microspheres. However, the microspheres that had been formed led to an interaction between the negatively charged polymer and the positively charged GDNF.

In Vitro GDNF Release

The release characteristics of GDNF from the poly (lactic-*co*-glycolic) acid (PLGA)-coated gelatin microspheres at different concentrations of GDNF were studied. The samples were immersed in 0.15*M* NaCl–PBS containing 0.1% BSA and incubated in a shaking water bath at 37°C with constant shaking at 40 rpm. The release profiles are given as the percentages of the cumulative amounts of GDNF released to the amounts of the drug actually loaded in the microspheres or to the actual weights of the microspheres, as presented in Figure 6.

The release profile of GDNF from microspheres at any time point was dependent on the amount of GDNF initially loaded within the microspheres. Thus, the highest amounts of GDNF (10 ng/mL) exhibited the highest amounts of GDNF released from the microspheres. The amount of GDNF released from these materials after immersion in the medium for 5760 min were about 86, 80, 70, 58, and 53% of the amounts of GDNF actually contained within microspheres that had been prepared with a concentration of GDNF of 0.625–10 ng /mL. However, all *in vitro* release profiles demonstrated a biphasic modulation. The first phase was characterized by a relatively rapid initial release and followed by a second slower phase.[32]

Cytotoxicity

The cytotoxicity of the microspheres was evaluated by estimating the cells viabilities of the neural stem cells. The MTT assay was used to measure the metabolic activity of the neural stem cells that had been cultured in the extraction medium of neat gelatin microspheres that had been incubated for 24 h as the control condition, by which the viabilities of the cells that had been cultured in the extraction media of the neat and the GDNF-loaded gelatin microspheres that had been incubated for all time intervals were normalized. The results in Figure 7 show that the relative viabilities of the cells that had been cultured in the extraction media of the neat gelatin microspheres that had been incubated for 48 and 72 decreased, but were still greater than the cut-off value of 80%, suggesting that could be some harmful substances, mostly likely unreacted GTA,[37] that were leashed out from the neat gelatin microspheres during the incubation. Interestingly, the relative viabilities of the cells that had been cultured in the extraction media of the GDNF-loaded gelatin microspheres were all greater than 100%. This should be a result of the presence of GDNF that had been leashed into the extraction media during the incubation periods that promoted the cell growth.

In Vitro Bioactivity Studies

The delivery system of GDNF must preserve the protein throughout the biological process. The evaluation of the bioactivity of the GDNF released from microspheres was performed

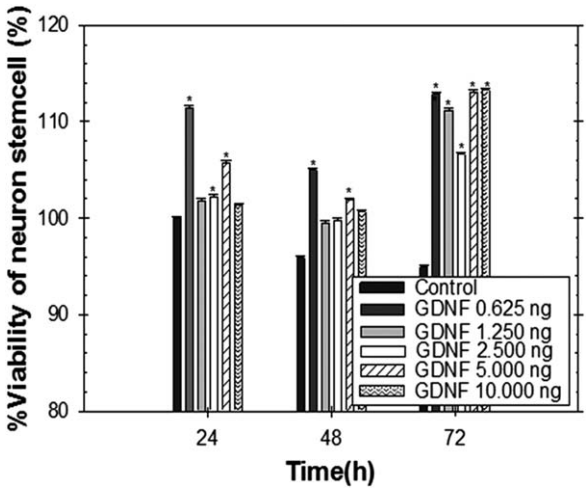

Figure 7. The viability of neural stem cells measured by the reduction of MTT ($n = 3$). The microspheres were more than 100% active when compared with the control group (24 h); these results can also be interpreted as showing that the microspheres are biocompatible. * is significantly different at $P < 0.05$; compared with the control group at any given time point.

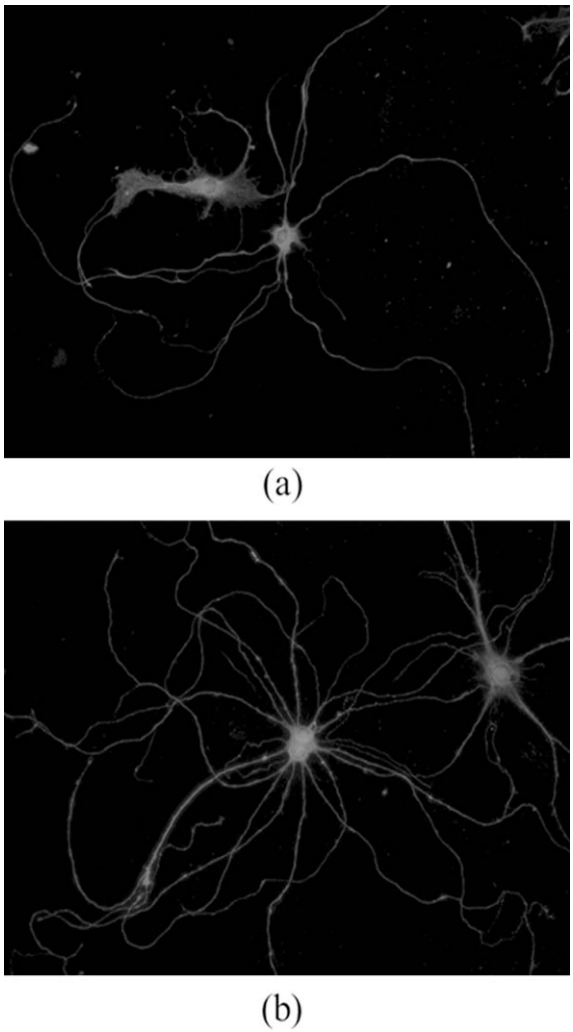

Figure 8. The bioactivity of GDNF released in TGCs. Immunofluorescence picture of cells that were incubated for 5 days in medium supplemented with the release medium of non-loaded microspheres (a) and with GDNF released from microspheres after 5 d (b) ($n = 50$). The TGCs treated with GDNF released from the microspheres demonstrated more robust neurite extension than non-loaded microspheres. The microspheres containing GDNF is significantly different at $P < 0.05$; compared with the control group.

in TGCs. Figure 8 shows that, the neurite length of the non-loaded microspheres (control) and the microspheres containing GDNF was 1660 ± 511 and 2290 ± 548 μm, respectively. The increase in neurite outgrowth of the TGCs was increased by the GDNF released from microspheres at day 5. Thus, these results show that the release of microspheres containing GDNF was biologically active.

CONCLUSIONS

The results of this study indicated that the gelatin type and the pH level influence the size of the microspheres, the surface charge, and the swelling ability. The microspheres were able to encapsulate GDNF due to the ionic interaction between the biodegradable polymers and the molecules of GDNF. Furthermore, the *in vitro* studies showed that the microspheres could be used to provide sustained release of the GDNF for at least 4 d *in vitro*. The microspheres prepared using a thermal gelation technique were non-toxic to the neural stem cells. The GDNF released from microspheres in TGCs (nerve cells) was bioactive. It has been shown that either 4-days pretreatment with GDNF or continuous supply of exogenous GDNF via osmotic pump after transplantation could promote survival and function of fetal ventral mesencephalic tissue transplants in the rat model of Parkinson's disease. Biodegradable GDNF microspheres, in theory, should promote graft survival while does not require high concentration of neurotrophic factors as infusion pump method.[38,39] Various GDNF microspheres with different materials and release profiles were previously reported. Next, it would be important to understand how the rate of biomaterials degradation, the level and length of GDNF release could affect clinical outcome and side effects after transplantation.[40,41] Further in vivo study comparing the effect of these microspheres on co-transplanted dopaminergic neurons is required to solve this issue.

ACKNOWLEDGMENTS

The authors acknowledge partial support received from (a) the Thailand Research Fund (TRF, grant no. DBG 52800), (b) the "Integrated Innovation Academic Center: IIAC(RES_01_54_63)," Chulalongkorn University Centenary Academic Development Project, Chulalongkorn University, (c) the Commission on Higher Education, Thailand, (d) the Center of Excellence on Petrochemical and Materials Technology (PETROMAT), (e) the Petroleum and Petrochemical College (PPC), Chulalongkorn University, (f) the Stem Cell and Cell Therapy Research Unit, Faculty of Medicine, Chulalongkorn University, and (g) the Neuroscience of Headache Research Unit, the Faculty of Medicine, Chulalongkorn University.

REFERENCES

1. Saltzman, W.; Baldwin, S. P. *Adv. Drug Delivery Rev.* **1998**, *33*, 71.
2. Li, D. C.; Zhong, X. K.; Zeng, Z. P.; Jiang, J. G.; Li, L.; Zhao, M. M.; Yang, X. Q.; Chen, J.; Zhang, B. S.; Zhao, Q. Z.; Xie, M. Y.; Xiong, H.; Deng, Z. Y.; Zhang, X. M.; Xu, S. Y.; Gao, Y. X. *J. Control Release* **2009**, *138*, 103.
3. Gupta, A. K.; Gupta, M.; Yarwood, S. J.; Curtis, A. S. G. *J. Control Release* **2004**, *95*, 197.
4. Wang, X.; Wenk, E.; Hu, X.; Castro, G. R.; Meinel, L.; Wang, X.; Li, C.; Merkle, H.; Kaplan, D. L. *Biomaterials* **2007**, *28*, 4161.
5. Ma, G.; Gong, F.; Hu, G.; Hao, D.; Liu, R.; Wang, R. *China Particuol.* **2005**, *3*, 296.
6. Kimura, Y.; Ozeki, M.; Inamoto, T.; Tabata, Y. *Biomaterials* **2003**, *24*, 2513.
7. Kawai, K.; Suzuki, S.; Tabata, Y.; Ikada, Y.; Nishimura, Y. *Biomaterials* **2000**, *21*, 489.
8. Holland, T. A.; Tabata, Y.; Mikos, A. G. *J. Control Release* **2003**, *91*, 299.
9. Kommareddy, S.; Amiji, M. *Nanomedicine* **2007**, *3*, 32.
10. Shen, H.; Hu, X.; Yang, F.; Bei, J.; Wang, S. *Biomaterials* **2011**, *32*, 3404.

11. DeFail, A. J.; Chu, C. R.; Izzo, N.; Marra, K. G. *Biomaterials* **2006**, *27*, 1579.
12. Curcio, M.; Gianfranco Spizzirri, U. G.; Iemma, F.; Puoci, F.; Cirillo, G.; Parisi, O. I.; Picci, N. *Eur. J. Pharm. Biopharm.* **2010**, *76*, 48.
13. Ikada, Y.; Tabata, Y. *Adv. Drug Delivery Rev.* **1998**, *31*, 287.
14. Strauss, G.; Gibson, S. M. *Food Hydrocolloids* **2004**, *18*, 81.
15. Haroun, A. A.; El-Halawany, N. R. *IRBM* **2010**, *31*, 234.
16. Kriegel, C.; Amiji, M. *J. Control Release* **2011**, *150*, 77.
17. Ferreira Almeida, P.; Almeida, A. J. *J. Control Release* **2004**, *97*, 431.
18. Hariraksapitak, P.; Development of Protein Delivery Scaffolds with a Separate Drug Carrier System for Bone Tissue Regeneration; Master's Thesis, Chulalongkorn University: Bangkok, Thailand, **2009**.
19. Nair, L. S.; Laurencin, C. T. *Prog. Polym. Sci.* **2007**, *32*, 762.
20. Morita, T.; Horikiri, Y.; Suzuki, T.; Yoshino, H. *Int. J. Pharm.* **2001**, *219*, 127.
21. Tan, H.; Wu, J.; Lao, L.; Gao, C. *Acta Biomater.* **2009**, *5*, 328.
22. Nojehdehian, H.; Moztarzadeh, F.; Baharvand, H.; Nazarian, H.; Tahriri, M. *Colloids Surf. B Biointerf.* **2009**, *73*, 23.
23. Faisant, N.; Akiki, J.; Siepmann, F.; Benoit, J. P.; Siepmann, J. *Int. J. Pharm.* **2006**, *314*, 189.
24. Wood, M. D.; Gordon, T.; Kemp, S. W.; Liu, E. H.; Kim, H.; Shoichet, M. S.; Borschel, G. H. *Biotechnol Bioeng.* **2013**, *110*, 1272.
25. Checa-Casalengua, P.; Jiang, C.; Bravo-Osuna, I.; Tucker, B. A.; Molina-Martínez, I. T.; Young, M. J.; Herrero-Vanrell, R. *J. Int. J. Pharm.* **2012**, *436*, 545.
26. Carnicella, S.; Ron, D. *Pharmacol. Ther.* **2009**, *122*, 9.
27. Eggert, K.; Schlegel, J.; Oertel, W.; Würz, C.; Krieg, J. C.; Vedder, H. *Neurosci. Lett.* **1999**, *269*, 178.
28. Wong, H. L.; Wang, M. X.; Chanug, P. T.; Yao, K. M.; Chan, B. P. *Biomaterials* **2007**, *28*, 5369.
29. Garbayo, E.; Montero-Menei, C. N.; Ansorena, E.; Lanciego, J. L.; Aymerich, M. S.; Blanco-Prieto, M. J. *J. Control Release* **2009**, *135*, 119.
30. Clavreul, A.; Sindji, L.; Aubert-Pouëssel, A.; Benoît, J. P.; Menei, P.; Montero-Menei, C. N. *Eur. J. Pharm. Biopharm.* **2006**, *63*, 221.
31. Tan, H.; Huang, D.; Lao, L.; Gao, C. *J. Biomed. Mater. Res., Part B.* **2009**, *91*, 228.
32. Garbayo, E.; Ansorena, E.; Lanciego, J. L.; Aymerich, M. S.; Blanco-Prieto, M. *J. Eur. J. Pharm. Biopharm.* **2008**, *69*, 844.
33. Vandervoort, J.; Ludwig, A. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 251.
34. Mladenovska, K.; Klisarova, L.; Kumbaradži, E.; Dodov, M.; Ivanova, E.; Goračinova, K. *Bull. Chem. Technol. Maced.* **2001**, *20*, 151.
35. Young, S.; Wong, M.; Tabata, Y.; Mikos, A. G. *J. Control Release* **2005**, *109*, 256.
36. Kushibiki, T.; Tomoshige, R.; Iwanaga, K.; Kakemi, M.; Tabata. Y. *J. Control Release* **2006**, *112*, 249.
37. Van Miller, J. P.; Hermansky, S. J.; Losco, P. E.; Ballantyne, B. *Toxicology* **2002**, *175*, 177.
38. Yurek, D. M. *Exp. Neurol.* **1998**, *153*, 195.
39. Nevalainen, N.; Chermenina, M.; Rehnmark, A.; Berglöf, E.; Marschinke, F.; Strömberg, I. *Neuroscience.* **2010**, *171*, 1357.
40. Herrán, E.; Ruiz-Ortega, J. A.; Aristieta, A.; Igartua, M.; Requejo, C.; Lafuente, J. V.; Ugedo, L.; Pedraz, J. L.; Hernández, R. M.; *Eur. J. Pharm. Biopharm*, **2013**, In press. (DOI: S0939-6411(13)00124-0)
41. Garbayo, E.; Ansorena, E.; Lanciego, J. L.; Blanco-Prieto, M. J.; Aymerich, M. S. *Mov. Disord.* **2011**, *26*, 1943.