Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres

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Abstract

Glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factor for dopaminergic neurons, appeared as a promising candidate for treating Parkinson's disease. GDNF microencapsulation could ensure protection against degradation due to the fragile nature of the protein. Poly(lactide-co-glycolide) (PLGA) microparticles loaded with recombinant glycosylated GDNF obtained in a mammalian cell line were prepared by TROMS, a semi-industrial technique capable of encapsulating fragile molecules maintaining their native properties. The effects of several parameters as PLGA copolymer type, PEG 400 quantity co-encapsulated with GDNF or drug loading, on the properties of the particles were investigated. Microparticles showed a mean diameter between 8 and 30 µm, compatible with their stereotaxic implantation. The drug entrapment efficiency ranged from 50.6 to 100% depending on the microsphere composition. GDNF was better encapsulated using hydrophilic polymers with high molecular weight such as RG 503H. *In vitro* drug release was influenced by the polymer type as well as by the amount of PEG 400 co-encapsulated with GDNF. Microparticles prepared using PLGA RG 503H released 67% of the total protein content within 40 days. Moreover, very low concentrations of poly (vinyl alcohol) were detected after microparticles washing and freeze-drying. Finally, a PC-12 bioassay demonstrated that the *in vitro* GDNF released was bioactive.

Key words: Rat recombinant GDNF, biodegradable microparticles, PLGA, TROMS, PEG 400

1. Introduction

Therapeutic proteins are a new type of drug that represent a growing sector in the drug market [1]. Numerous therapeutic proteins have been approved or are in advanced clinical testing [2]. In particular, a total of 16 new therapeutic proteins were approved by the FDA during 2004 and 2005 [3]. In contrast to other therapeutic agents that are normally used, these macromolecules have high specificity and activity at relatively low level concentrations. However, protein administration presents other serious limitations due to their short *in vivo* half life, physical and chemical instability and low oral bioavailability [4]. Several years ago, the use of microspheres as carriers of these compounds was proposed as an attractive way to overcome these problems. Microspheres would offer several advantages since they could provide protein protection from degradation and a prolonged delivery. The encapsulation of a wide range of proteins has been studied, although the development of delivery systems for protein as drugs is still a major challenge [5]. Among the methods described to prepare microparticles, multiple emulsion solvent evaporation technique (W/O/W) is widely accepted as the most suitable for encapsulation of labile hydrophilic compounds such as peptides and proteins [6]. However, there are two critical steps in this process that could affect the protein activity; shear stress and the water/organic interface. Microsphere preparation by Total Recirculation One-Machine System (TROMS) could provide an appropriate alternative to avoid the first aspect. This system, based on the injection of the phases under a turbulent regime, does not need vigorous agitation to prepare the microspheres [7]. Thus, shear stress is avoided and proteins could remain active. Moreover, the use of protein stabilizers such as sugars, proteins, polyols or metals, prevents the denaturalization of the protein in the w/o interface [6].

Among the biodegradable polymers, the copolymers of lactic and glycolic acids (PLGAs) have become widely used for therapeutic protein delivery due to their excellent biodegradability and biocompatibility [8]. Numerous proteins have successfully been encapsulated into PLGA microspheres such as erythropoietin [9], growth hormone [10], interferon gamma [10], calcitonin [11], chorionic gonadotrophin [12], nerve growth factor [13], brain derived neurotrophic factor [14] or glial cell-line derived neurotrophic factor (GDNF) [15] among others.

GDNF, the protein used in this study, has been described as one of the most potent neurotrophic factors for dopaminergic neurons with both neuroprotective and neurorestorative properties [16]. In addition, it stimulates regenerative growth and axonal sprouting in animal models of Parkinson's disease (PD) [17]. Two open-label trials involving continuous recombinant nonglycosylated GDNF infusion into the putamen of PD patients showed that the growth factor significantly improved motor scores as measured with the Unified Parkinson's disease Rating Scale (UPDRS) [18-20]. However, a double-blind placebo controlled study presented by AMGEN did not demonstrate beneficial effects in patients [21, 22]. Differences in doses, catheter design and delivery protocols may be responsible for the discrepancy between phase I and II studies. Clearly, alternative methods of GDNF delivery to the brain must be developed. In this sense, polymer-based drug delivery systems could be a valuable strategy.

In the present work, the preparation and characterization of PLGA microspheres loaded with highly pure recombinant glycosylated GDNF obtained in a mammalian cell line are described. Particles were prepared by solvent evaporation technique using TROMS technology. The effects of several formulation parameters such as PLGA copolymer type, PEG 400 quantity co-encapsulated with GNDF and drug loading on

particle size, encapsulation efficiency and *in vitro* release kinetics were analyzed. Attention was also focused on the bioactivity of the protein.

2. Materials and Methods

2.1 Materials

Rat recombinant GDNF was expressed and purified as previously described [23]. GDNF enzyme linked immunosorbant assay kit (ELISA) was purchased from Promega (Madison, USA). Poly(lactic-co-glycolic) acid (PLGA) with a lactic : glycolic ratio of 50:50 Resomer RG 503 (MW 34 kDa), Resomer RG 502H (MW 13.7 kDa) and Resomer RG 503H (MW 34 kDa) were provided by Boehringer-Ingelheim (Ingelheim, Germany). Poly(ethylene glycol) 400 (PEG 400) and human serum albumine (HSA) were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A (Barcelona, Spain). Poly (vinyl alcohol) (PVA) 88% hydrolyzed (MW: ~ 125,000) was obtained from Polysciences, Inc (Warington, USA). The adrenal rat PC-12 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). Silver Stain Plus was obtained from BioRad (California, USA). General laboratory reagents were purchased from Sigma-Aldrich (Barcelona, Spain) unless specified in the text.

2.2 Protein purity assay

SDS-PAGE and silver staining were performed to assess GDNF purity. Purified rat recombinant GDNF was loaded onto 12.5% polyacrylamide gels under reducing conditions. After the electrophoresis, gels were stained with Silver Stain Plus according to manufacturer's instructions.

2.3 Microparticles preparation

Microparticles containing purified rat recombinant GDNF were prepared by solvent extraction/evaporation method using Total Recirculation One-Machine System

(TROMS) [7]. Briefly, the organic solution composed of 2 ml of dichlorometane:acetone (3:1) containing the polymer was injected through a needle with an inner diameter of 0.17 mm at 30 ml/min into the inner aqueous phase. The inner water phase contained rat recombinant GDNF in 10 mM phosphate, 50 mM sodium chloride (PBS), pH 7.9, 5% of HSA and different quantities of PEG 400. Next, the previously formed inner emulsion (W_1/O) was recirculated through the system for 3 min under a turbulent regime at a flow rate of 30 ml/min. After this step, the first emulsion was injected into 30 ml of an aqueous phase (W_2) composed of 1.5% PVA. The turbulent injection through the needle with an inner diameter of 0.50 mm resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was further homogenized by circulation through the system for 4 min. The resulting $W_1/O/W_2$ emulsion was stirred for at least 3 h at room temperature. Microparticles were washed three times with ultrapure water by consecutive centrifugation at 4 ºC (20000 g, 10 min.). Finally, the particles were resuspended in 1 ml of ultrapure water, frozen at -80 ºC, lyophilized (Genesis 12EL, Virtis) and stored at 4 ºC.

2.4 Microparticle characterization

2.4.1 Particle size

Size and size distribution of the microspheres were determined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). Microspheres were dispersed in distilled water and analyzed under continuous stirring. The results were expressed in volumetric mean diameter, which is the diameter that divides the volume distribution curve of the sampled microparticles in two equal parts. Samples were measured in triplicate.

2.4.2 Morphology

Morphology of the microspheres with highest drug loadings was analyzed by scanning electron microscopy (SEM). The lyophilized particles were directly deposited onto a carbon conductive tape on aluminium stubs and coated with gold to a 16 mm thickness (Emitek K550 equipment). The samples were observed in a Zeiss DSM 940A microscope with a digital imaging capture system (DISS of Point Electronic GmbH, Halle, Germany).

2.4.3 Determination of residual PVA

The amount of PVA associated with microparticles was determined by a colorimetric method based on the formation of a coloured complex between two adjacent hydroxyl groups of PVA and an iodine molecule [24, 25]. Briefly, 2 mg of lyophilized microparticles samples were treated with 2 ml of 0.5 M NaOH for 15 min at 60 ºC. Each sample was neutralized with 900 µl of 1 N HCl and the volume was adjusted to 5 ml with distilled water. To each sample, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M) and 1.5 ml of distilled water were added. Finally, the absorbance of the samples was measured at 690 nm using an Agilent 8453 UV-Visible spectrophotometer (Agilent technologies, Palo Alto, CA, USA) after 15 min of incubation. A standard plot of PVA was prepared under identical conditions.

2.4.4 Determination of GDNF content in the microparticles

To quantify the GDNF content in the microparticles, 1 ml of dimethyl sulfoxide (DMSO) was added to 5 mg of freeze-dried loaded particles. Previously, it was verified that DMSO did not affect GDNF stability. The mixture was vortexed vigorously for 5 min and the amount of GDNF was measured by ELISA. Briefly, 96-well microplates

(Greiner Bio-One Germany) were coated with monoclonal anti-GDNF antibody diluted in 0.025 M carbonate buffer pH 8.2 and incubated overnight at 4 ºC. Plates were blocked for 1 h with blocking solution provided by the manufacturers. The samples were added to the coated wells (100 µl each) and incubated for 6 h at room temperature. Plates were washed 5 times and bounded GDNF was incubated with anti-GDNF polyclonal antibody overnight at 4 ºC. After additional washed, plates were incubated for 2 h at room temperature with anti-chicken IgY, HRP conjugate. Then, plates were washed 5 times and incubated with TMB/peroxidase substrate solution for 15 min. The enzyme reaction was stopped by adding an acidic solution. Optical density was determined in a plate reader set at 450 nm. Sample values were determined from the regression standard line for the purified rat GDNF (ranging from 15 to 1000 pg/ml) prepared for each assay. Points of the regression standard line were treated with the same amount of DMSO as the samples. Each sample was assayed in triplicate. Drug entrapment efficacy was calculated as the ratio of the final drug content in the microspheres to the initial drug content, expressed as a percentage.

2.5 In vitro release of GDNF

GDNF-loaded microparticles, accurately weighed (1 mg, n = 3), were incubated in 0.5 ml of 10 mM phosphate, 150 mM sodium chloride, pH 7.4 containing 0.1% BSA and 0.02% w/w sodium azide. Incubation took place in rotating vials at 37 ºC for 1 week or 40 days depending on the experiments. Due to the instability of the protein in the release medium, the amount of drug released was determined indirectly by measuring the amount of GDNF remaining in the microspheres. At defined time intervals, sample tubes were centrifuged (25000 *g*, for 15 min). After removal of the supernatant, microspheres were dissolved with DMSO and the protein content was

determined by ELISA as described above. Release profiles were expressed in terms of cumulative release, and plotted *versus* time.

2.6 In vitro bioactivity assay

The differentiation of PC-12 cells was used to evaluate the bioactivity of GDNF released from microparticles. These cells differentiate to a neuronal phenotype extending neurites in response to neurotrophic factors such as NGF or GDNF [23, 26]. PC-12 cells were cultured in D-MEM supplemented with 5% horse serum, 10% foetal bovine serum and 1% penicillin/streptomycin. For studies of neurite outgrowth, PC-12 cells were plated onto 12 well culture plate at a low density, 2 x 10^3 cells/cm^2 in 1 ml of culture media. The culture medium was supplemented 24 hours later with 50 ng of GDNF released from microspheres over 24 hours, which had previously been quantified by ELISA. Neurite outgrowth was visualized after 7 days in culture under phase contrast illumination with a Leika DM IRB inverted microscope connected to a Hamamatsu ORCA-ER digital camera. PC-12 cells incubated with 50 ng/ml of purified rat recombinant GDNF were used as a positive control of the technique. The released medium from non-loaded microspheres was used as negative control for the experiment.

3. Results and discussion

3.1 Protein purity assay

A GDNF expression and purification procedure had previously been developed in order to obtain bioactive, highly pure and glycosylated protein [23]. Since GDNF was intended to be used for *in vivo* studies, testing the purity of the protein was considered crucial. Rat recombinant GDNF was analyzed by SDS-PAGE and posterior silver staining. Silver staining showed a highly pure protein (Figure 1). GDNF migrated as a 26 kDa band that corresponds to the most abundant glycosylated form of the protein. Highly pure protein was obtained, in sufficient amounts to be microencapsulated. This was an important aspect, since impurities could affect not only the encapsulation efficiency and the release profile of the neurotrophic factor but also the *in vivo* efficacy. Thus, recombinant N-glycosylated GDNF, obtained in a mammalian cell line and similar to the endogenous protein, was used to formulate the microspheres.

3.2 Microsphere preparation and characterization

Several attempts were made to overcome the critical steps of microsphere preparation process: shear stress and water/organic interface. Attention was focused on the preservation of the protein biological activity. In this study, several formulations containing rat recombinant glycosylated GDNF were prepared by emulsion solvent evaporation technique using TROMS (Table 1). This procedure avoids shear stress produced by sonication and ultraturrax that normally would affect protein integrity and consequently, their biological activity [7]. Garcia del Barrio *et al.,* compared the activity of a labile compound such as adenovirus after its microencapsulation by conventional multiple emulsion solvent evaporation technique and by TROMS. Microparticles prepared by TROMS presented 4.5 and 3 times more activity at 8 and 32 h than

microparticles prepared by the conventional methods [27]. Another advantage of TROMS over microparticle production through emulsion methods is the ability to produce homogeneous batches on a semi-industrial scale. Consequently, this would be of great interest considering scaling-up and industrial issues.

HSA and PEG 400 were co-encapsulated with GDNF in order to stabilize the primary emulsion and to reduce protein-polymer interactions. Previous studies showed that PEG 400 dissolved in the inner aqueous phase was a good candidate to protect NGF against denaturing by contact with an organic phase during emulsification without modifying the microparticle structure [28]. In this case, PEG 400 limited protein penetration in the interfilm of the primary emulsion and reduced the contact between the protein and the organic phase. Furthermore PEG 400 promoted sufficient release of entrapped NGF. Subsequently, this compound was co-encapsulated with insulin like growth factor I [29], L-asparaginase enzyme [5] or non glycosylated GDNF [30] to prepare particles by conventional multiple emulsion solvent evaporation method.

3.2.1 Particle size and morphological analysis

Particle size was measured by laser diffractometry. All the microparticles exhibited a monomodal size distribution with a mean diameter between 8.4 µm and 30.9 µm, compatible with a stereotaxic injection (Table 1). The mean particle size decreases when the nominal drug loading increases. Thus, at nominal GDNF content of 0.5 µg, the mean particle size was around 29 µm independently of the polymer used. Surprisingly, when 135 µg of GDNF were included in the inner water phase, the mean particle size decreased to 8.4 µm. Non-loaded microspheres prepared under the same conditions showed a mean particle size of 30 µm. The decrease in microparticle size could be ascribed to tensioactive properties of the protein.

The mean particle size was slightly influenced by the addition of PEG. As shown in Table 1, when the concentration of PEG in the inner water phase was increased up to 10%, a decrease in the particle size from 27.7 µm to 20.4 µm was observed.

Figure 2 shows the surface morphology of the microparticles prepared with polymer RG 503H and loaded with 135 µg of GDNF. SEM revealed that GDNF-loaded microparticles had spherical shapes with a smooth surface on which a few small pores were visible in some particles.

3.2.2 Residual PVA

PVA is normally used as emulsifier in the formulation of PLGA microspheres. This polymer prevents microsphere coagulation during solvent removal. It is described in the literature that a fraction of PVA remains associated with the particles despite repeated washing [25]. However, as PVA is a potentially toxic non-biodegradable polymer, its administration should be minimized as much as possible [31, 32]. The amount of residual PVA was quantified in different batches of microspheres. Very low concentrations of PVA (ranging from 0.7 to 1.3%) were detected in all the formulations analyzed, after washing and freeze-drying of the particles. These concentrations are up to five times lower than the reported in the literature for PLA microparticles [33].

3.2.3 GDNF encapsulation efficiency

The amount of rat recombinant GDNF encapsulated was determined by ELISA. The neurotrophic factor was efficiently encapsulated independently of the polymer used (Table 1). However, some differences were highlighted between the formulations. At a constant nominal loading of 0.5 µg of GDNF, RG 503H microspheres showed the highest encapsulation value (100%), followed by polymers RG 502H and RG 503 that

presented similar percentages (78.2% and 79.7% respectively). On the contrary, the blend of polymers RG 502H and RG 503H (1:1, w/w) exhibited the lowest encapsulation rate (69.7%). Thus, the highest entrapment efficacy was achieved using RG 503H, a hydrophilic polymer with high molecular weight (34 kDa). Hydrophilic compounds such us GDNF, are generally better encapsulated using hydrophilic polymers. This is probably due to molecular interactions between the drug and the polymer. Furthermore, RG 503H has a higher inherent viscosity than RG 502H (0.38 *vs* 0.19 dl/g). Consequently, the increase in the polymer solution viscosity led to a higher entrapment of the protein because it limits the migration of the protein from the inner phase towards the outer one [34].

Drug loading may also be influenced by the initial amount of drug introduced in the inner aqueous phase. The highest encapsulation efficiencies, ranging from 69.7 to 100%, were found at the lowest nominal drug loading (0.5 µg of GDNF). On the other hand, when nominal GDNF content was increased up to 135 µg, the protein loading in the microspheres increased (680 ng/mg polymer) while the entrapment efficacy decreased to 50.6%. This is a well described phenomenon that affects water soluble drugs and is probably related to the high osmotic pressure and the large pores formed at high loadings [35].

As can be seen in Table 1, the encapsulation efficiencies were not affected by the co-encapsulation of different percentages of PEG with GDNF. This is consistent with a previous report that showed no influence of co-encapsulation of PEG with NGF on the neurotrophic factor encapsulation efficiencies [28].

3.3. In vitro release of GDNF

The cumulative *in vitro* release profile of GDNF from microparticles prepared with different PLGA polymers is shown in Figure 3. All the formulations showed a low initial burst effect due to the release of GDNF adsorbed on the particle surface. The amount of GDNF released during the first 8 hours was dependent on the polymer molecular weight. Thus, the highest molecular weight polymers (RG 503H and RG 503) exhibited highest initial burst (8.9% and 8.7% respectively). On the other hand, the blend of Resomer RG 502H and RG 503H released 6.3% and Resomer RG 502H released 5.7% of the total dose. Positively charged molecules could potentially interact with PLGA negatively charged carboxylic end groups [36]. This could be the case for GDNF, since it has a pI of 9.44 and it is positively charged at pH 7.9, the inner water phase pH. Such interactions could explain the faster drug release observed at higher polymer molecular weights [36]. After one week, GDNF released from PLGA microspheres were not strongly affected by polymer molecular weight. Microparticles prepared with RG 503, RG 502H and the blend of RG 503H and RG 502H showed a similar cumulative drug release (11.1%, 11.6% and 11.4% respectively). However, RG 503H microspheres released GDNF slightly faster and 13.5% of the drug was released during the first week. Thus, taking into account the highest encapsulation efficiencies values and the GDNF *in vitro* release kinetics, the copolymer RG 503H was selected for further studies.

The incorporation of additives to the formulation can substantially modify the drug release profile. Since PEG 400 was co-encapsulated with GDNF to protect the protein biological activity, the effect of PEG on GDNF release profile from RG 503H microparticles was investigated (Figure 4). All the formulations studied showed a biphasic release profile characterized by an initial burst release phase followed by a slower drug release phase. The co-encapsulation of PEG with GDNF influenced the

initial release and the amount of released protein over a 7 day period but did not change the biphasic drug release pattern (Figure 4). As shown in Figure 4, during the first 8 hours microspheres containing 1% of PEG yielded an initial release of 20% as compared to 34.3% and 41.8% observed for the formulations containing 5 and 10% of PEG respectively. A similar effect was observed during the release of NGF from PLGA microspheres [28]. This effect could be due to the inhibition of protein-polymer adsorption and to the improvement of polymer solubility [28]. Following the initial burst, up to 27.5% (1% PEG), 45.8% (5% PEG) or 52.4% (10% PEG) of total GDNF was released throughout one week (Figure 4). Among the different percentages of PEG analyzed, microspheres prepared with 5% and 10% provided the best release characteristics over one week, for the tested conditions (Figure 4). Taking into consideration that the final aim of this project will be to administer the microparticles into the brain of animal models, microparticles prepared with a lower amount of PEG were selected and the release of GDNF was studied over 40 days. The release of the protein was biphasic. After an initial burst caused by the release of GDNF adsorbed on the particle surface, a sustained release was observed from day 1 to day 14, in which drug diffuses through the polymer. Finally, an increase in the rate of release was observed from day 14 to 40 due to polymer degradation and, 67% of the total GDNF was released within the first 40 days. Other authors have previously noticed a correlation between increased protein release and polymer degradation [26].

Besides the polymer type, and the incorporation of additives, the initial release could also be affected by the protein loading. Since the initial release is normally attributed to the surface-associated drug, higher protein loading led to a higher amount of drug located close to the particle surface and, in consequence, to an increase in the initial burst. In this sense, it was observed that the initial GDNF released during the first

8 hours increased from 8.9 to 20% when the protein loading increased from 0.5 to 20 µg for RG 503H microparticles containing 1% of PEG . These results are in agreement with the results obtained previously by other authors [37-39].

3.4 In vitro bioactivity assay

Since the successful development of a delivery system for GDNF requires the preservation of the protein biological function throughout all the process, the bioactivity and stability of released GDNF were evaluated *in vitro* using a PC-12 differentiation assay. The protein released from microspheres over 24 h was added to the culture medium of the cells. This cell line responds to bioactive GDNF by differentiating to a neuronal-like phenotype that is visualized by the sprouting of neurites. After one week of exposure to the neurotrophic factor, PC-12 cell neurite outgrowth indicated that released GDNF was bioactive (Figure 5 B). A similar effect was observed in cells treated with purified rat recombinant GDNF (Figure 5C). On the contrary, no outgrowth could be seen in PC-12 cells incubated with the release medium from unloaded microspheres. In this case, cells presented an undifferentiated and rounded morphology (Figure 5A). These results demonstrated that the encapsulated GDNF was biologically active. Indeed, GDNF bioactivity was maintained in the microspheres for at least 5 weeks (data not shown). Since proteins may lose their bioactivity during microparticle preparation and posterior release, the assurance that the biological activity of GDNF was preserved would appear to be extremely important in the perspective of microsphere implantation in animal models of PD.

4. Conclusions

The results of this study show that rat recombinant glycosylated GDNF obtained in a mammalian cell line can be successfully microencapsulated in PLGA microspheres by TROMS technology. Microparticles were able to release glycosylated GDNF in a controlled manner for at least 40 days *in vitro*. Moreover, the encapsulated GDNF was biologically active and could stimulate PC-12 cells to sprout neurites. Our results demonstrated that the released GDNF protein exhibited similar potency to naked purified GDNF protein in differentiating the PC-12 cells. At present, selected formulations are under *in vivo* evaluation in an animal model of Parkinson's disease.

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