Preservation of biological activity of glial cell line-derived neurotrophic factor (GDNF) after microencapsulation and sterilization by gamma irradiation

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A B S T R A C T
A main issue in controlled delivery of biotechnological products from injectable biodegradable microspheres is to preserve their integrity and functional activity after the microencapsulation process and final sterilization. The present experimental work tested different technological approaches to maintain the biological activity of an encapsulated biotechnological product within PLGA [poly (lactic-co-glycolic acid)] microspheres (MS) after their sterilization by gamma irradiation. GDNF (glial cell line-derived neurotrophic factor), useful in the treatment of several neurodegenerative diseases, was chosen as a labile model protein. In the particular case of optic nerve degeneration, GDNF has been demonstrated to improve the damaged retinal ganglion cells (RGC) survival.

GDNF was encapsulated in its molecular state by the water-in-oil-in-water (W/O/W) technique or as solid according to the solid-in-oil-in-water (S/O/W) method. Based on the S/O/W technique, GDNF was included in the PLGA microspheres alone (S/O/W 1) or in combination with an antioxidant (vitamin E, Vit E) (S/O/W 2). Microspheres were sterilized by gamma-irradiation (dose of 25 kGy) at room and low (−78 °C) temperatures. Functional activity of GDNF released from the different microspheres was evaluated both before and after sterilization in their potential target cells (retinal cells).

Although none of the systems proposed achieved with the goal of totally retain the structural stability of the GDNF-dimer, the protein released from the S/O/W 2 microspheres was clearly the most biologically active, showing significantly less retinal cell death than that released from either W/O/W or S/O/W 1 particles, even in low amounts of the neurotrophic factor.

According to the results presented in this work, the biological activity of biotechnological products after microencapsulation and sterilization can be further preserved by the inclusion of the active molecule in its solid state in combination with antioxidants and using low temperature (−78 °C) during gamma irradiation exposure.

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1. Introduction

Encapsulation of biotechnological products within biodegradable polymers has been intended to avoid frequent administrations in the treatment of chronic diseases. Drug delivery systems such as biodegradable microspheres are promising therapeutic tools to provide sustained delivery of the active agent for long term treatments avoiding successive administrations. To date, however, there are still problems associated with protein stability after microencapsulation and sterilization that remain unresolved. The microencapsulation of biological products in polymeric matrix is compromised by the high molecular weight, structural complexity, easy degradation, and instability of these active molecules (Sinha and Trehan, 2003; Al Haushey et al., 2007; Hamishehkar et al., 2009; Yuan et al., 2009; Ye et al., 2010). The choice of an appropriate method to microencapsulate macromolecules is critical, not only because it influences the physical or technological properties of microspheres, but also because the selected method should ensure the integrity of the biotechnological product during the preparation and storage of the systems (Sinha and Trehan, 2003; Andreas et al., 2011). Furthermore, it is necessary to select an appropriate process to guarantee that the final sterilization of the microspheres will not negatively affect biological activity of the encapsulated biotechnological compounds.

Among the different sterilization procedures available, gamma irradiation has been extensively used for microspheres prepared with biodegradable polyester polymers such as PLGA (Hausberger...
et al., 1995; Montanari et al., 1998; Bittner et al., 1999; Faisant et al., 2002; Martínez-Sancho et al., 2004; Fernández-Carballedo et al., 2004; Dorati et al., 2008). However, it is well known that γ-radiation can induce structural changes in both the polymer and on the encapsulated drug, especially if the active molecule is a protein (Montanari et al., 1998; Jain et al., 2011). Irradiation can produce denaturation and degradation that affect the integrity and bioactivity of the therapeutic agent (Jain et al., 2011). Several groups have reported the effects of gamma irradiation on PLGA microspheres entrapping proteins and peptides (Shameem et al., 1999; Carrascosa et al., 2003; Schwach et al., 2003; Dorati et al., 2005; Puthli and Vavia, 2008; Igartua et al., 2008; Mohanan et al., 2012). One of the most frequent problems is the formation of free radicals and reactive oxygen species (ROS) from both PLGA chains and proteins due to ionization phenomena, which might promote non-desired events. Among them, oxidation of side-chain groups, protein scission, backbone fragmentation, and conformational changes of the loaded macromolecule are the most common. It is generally accepted that large proteins are more prone to the alterations induced by gamma irradiation than peptides (Mohanan et al., 2012).

Several strategies have been proposed to overcome the risk associated with ionizing radiation of microparticulate formulations. The use of low temperatures (dry ice) during the sterilization of microspheres loaded with low molecular weight drugs (i.e., aciclovir, ganciclovir, ibuprofen, and indomethacin) has demonstrated to undergo no changes in the properties of the formulation after gamma irradiation exposure (Martínez-Sancho et al., 2004; Fernández-Carballedo et al., 2004, 2006; Herrero-Vanrell et al., 2000). However, this is not the case of microspheres loaded with biological products in which the use of low temperatures during irradiation does not provide the complete protection against undesirable reactions (i.e., protein carbonylation and hydroperoxide generation) (Zbikowska et al., 2006). These findings indicate that the use of low temperatures as an isolated procedure is not enough to assure preservation of biotechnological products exposed to irradiation.

The removal of free radical intermediates or inhibition of other oxidation reactions produced by ionizing radiation, which can be achieved by antioxidants (Martínez-Sancho et al., 2004; Mohanan et al., 2012; Zbikowska et al., 2006), could also offer protection of the encapsulated compounds. In this sense, the use of antioxidants as additives in the microspheres loaded with biological products results very attractive.

Furthermore, the inclusion of the active agent in its solid form has been proposed as another strategy that could promote the drug stability during sterilization, maintaining the initial conformation and minimizing the structural changes, compared with the use of the biomacromolecule in its molecular state (Schwach et al., 2003).

To the best of our knowledge, the effects of gamma-irradiation in proteins microencapsulated in its solid state combined with antioxidants have not been reported in the literature yet.

The objective of the present study was the evaluation of three technological approaches targeted at retaining protein biological activity after microencapsulation and sterilization. GDNF, a dimeric protein of 15–20 kDa/monomer, was chosen as a labile model protein. GDNF has been proposed as a therapeutic agent in neuroprotection because of its both neuroprotective and neurorestorative properties (Klocker et al., 1997; Koeberle and Ball, 1998; Yan et al., 1999; Andrieu-Soler et al., 2005; Ward et al., 2007; Jiang et al., 2007). In the particular case of optic nerve degeneration, GDNF has been demonstrated to improve the damaged retinal ganglion cells (RGC) survival after its intravitreal injection emerging as a good candidate for the treatment of glaucomatous optic neuropathy (Ward et al., 2007; Jiang et al., 2007).

The biotechnological product (GDNF) was encapsulated in PLGA microspheres in its solid or in its molecular state. To this, GDNF was included in its molecular state using the solvent evaporation technique from a water-in-oil-in-water emulsion (W/O/W) and as solid employing the solid-in-oil-in-water emulsion (S/O/W). Based on the S/O/W technique, the protein was included in the presence (S/O/W 2) or absence of vitamin E (S/O/W 1). The inclusion of vitamin E in the microspheres (S/O/W 2) was tested as an approach to prevent radical induced degradation. Microspheres obtained from the different techniques were sterilized by gamma-irradiation (25 kGy) at room and low (−78 °C) temperatures. Loading efficiency, morphology, mean particle size, and particle size distribution, scanning electron microscopy (SEM), gel permeation chromatography (GPC) and in vitro release assays for 133 days were performed to evaluate the sterilization effect on microsphere characteristics. Functional activity of GDNF released from the different microspheres was evaluated both before and after sterilization in their potential target cells (retinal cells).

2. Materials and methods

2.1. Materials

Recombinant human GDNF and the ELISA (enzyme-linked immunosorbant assay) kit for GDNF quantification were supplied by R&D Systems (Minneapolis, MN, USA). Poly-(d,l-lactide-co-glycolide acid) PLGA ratio 50:50 Mrw 35,000 Da (Resomer® 503) was purchased from Boehringer Ingelheim (Pharma Co., Germany). Polyvinyl alcohol 72,000 g/mol (PVA) was supplied by Merck KGaA (Darmstadt, Germany). α-Tocopherol acetate (Vit E) and Bovine Serum Albumin (BSA) were obtained from Sigma–Aldrich (Schnelldorf, Germany). Primary antibody anti-GDNF and secondary antibody anti-goat for WB were purchased from R&D Systems and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. ECL Western Blotting Detection Kit was supplied by GE Healthcare (Little Chalfont, UK). C57BL6 mice were provided by Charles River Laboratories, Wilmington, MA. All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental protocols were approved by the Animal Care and Use Committee of the Schepens Eye Research Institute.

2.2. Methods

2.2.1. Microspheres elaboration

GDNF-loaded PLGA microspheres were prepared using three different emulsion–solvent extraction evaporation methods, W/O/W, S/O/W 1, and S/O/W 2 (including Vit E in the inner phase). For preparation of the primary W/O emulsion, 50 μl of the internal aqueous phase (PBS pH 7.4) containing 20 μg of recombinant human GDNF was emulsified with an organic solution composed of 1 ml of PLGA in methylene chloride (CH2Cl2) (20%, w/v). In the S/O/W 1 method, the S/O emulsion was performed by suspending 20 μg of recombinant human GDNF in 100 μl of methylene chloride and posterior mixing with 0.9 ml of PLGA/CH2Cl2 solution (22%, w/v). In the third method (S/O/W 2), 20 μg of recombinant human GDNF were suspended in 20 μl of Vit E and mixed with 1 ml of PLGA solution in methylene chloride (20%, w/v) (Checa-Casalengua et al., 2011). In all cases, the primary W/O emulsion and S/O suspensions were performed via sonication at low temperature (Sonicator XL, Head Systems, IA, USA). After that, the prepared organic phases were emulsified with 5 ml of PVA MilliQ® water solution (2%, w/v) in a homogenizer (Polytron® RECO, KinematicaGmbH PT 3000, Lucerna, Switzerland) at 5000 rpm for 1 min.

The formed emulsions were subsequently poured onto 100 ml of an aqueous PVA solution (0.1%, w/v) and were kept under
constant stirring for 3 h, to allow organic solvent evaporation. Once formed, microspheres were washed with MilliQ® water to eliminate the PVA and separated according to their particle size by filtration under vacuum conditions using 20 μm and 40 μm sieves. Particles were rapidly frozen (methanol/ice mixture) and freeze-dried to obtain a fine flowing powder. Microspheres were kept at −20 °C under dry conditions until use.

2.2.2. Microspheres characterization

Morphological evaluation: The morphology of microspheres was evaluated by scanning electron microscopy (SEM; Jeol, JSM-6335F, Tokyo, Japan). Samples were gold sputter-coated prior to observation.

Mean particle size and particle size distribution were measured by light scattering in a Microtrac® S3500 Series Particle Size Analyzer (Montgomeryville, PA, USA). Samples were prepared by suspending the microspheres in MilliQ® water. The data were presented as mean volume diameter ± SD of three independent measurements.

Encapsulation efficiency of GDNF: 5 mg of microspheres were placed in 0.7 ml of methylene chloride. Once the polymer was dissolved, the same volume of the diluent reactive provided in the ELISA kit composed by PBS 7.4 and BSA 1% (w/v) was added. After strong mixing, both immiscible phases were separated by centrifugation (7880 × g, 15 min). After separation, the aqueous phase was recovered. The so-performed liquid/liquid extraction was repeated four times, in order to recover the encapsulated protein. Assays were performed in triplicate. GDNF content in the aqueous medium was quantified by immunoassay.

In vitro release studies: 5 mg of microspheres were suspended in 1.5 ml of PBS pH 7.4 with BSA 1% (w/v) and sodium azide 0.02% (w/v). “Low binding” Eppendorf® vials were used in all cases. Samples were placed in a shaker with constant agitation at 100 rpm (Clifton Shaking Bath NE5, Nikel Electro Ltd., Avon United Kingdom) at 37 °C.

At pre-set times (1 h, 24 h, and once a week until the end of the assay) the microsphere suspensions were gently centrifuged (490 × g for 3 min), and the supernatant was recovered (approx. 1.5 ml) and replaced by the same volume of fresh medium to continue the release test. Supernatants were analyzed by the ELISA technique.

2.2.3. Polymer integrity

High-performance gel permeation chromatography (GPC): The molar mass of the polymer (PLGA) was evaluated by GPC at different times during the release assay (1 h, 4, 7, 12, and 19 weeks). Microspheres were dissolved in THF at concentrations ranging from 1 to 2 mg/ml. Before injection, samples were filtered using a PTFE membrane (0.2 μm). Flow rate was 1 ml/min of THF at 33 °C. 20 μl of the solution was injected. Two columns PLgel 3 μm MIXED-E and PLgel 5 μm MIXED-D, both of 7.8 mm × 300 mm (Varian, Polymer Laboratories, Church Stretton, Shropshire, UK), were connected consecutively to increase the accuracy of the procedure. The apparatus included a Waters 1525 binary HPLC pump and a Waters 2414 Refractive Index Detector (Waters, Saint-Quentin en Yvelines, France). The column was calibrated using polystyrene standards of different molar masses: 381, 1100, 2950, 6520, 18,600, and 43,700 g/mol; purchased from Waters (Mainz, Germany), 10,100, 24,600, and 72,450 g/mol, supplied by Varian (Polymer Laboratories) (Church Stretton, Shropshire, UK).

2.2.4. Sterilization process

Microspheres elaborated by the three microencapsulation methods were sealed in glass vials. All samples were irradiated using 60Co in the Gamma Sterilization Unit of Aragogamma S.A. (Barcelona, Spain). Following the USP recommendations, a dose of 25 kGy was applied to ensure an effective sterilization (Herrero-Vannelli et al., 2000). Microspheres were sterilized both at room temperature and in presence of dry ice (−78 °C), in order to determine the effect of the temperature during the irradiation process. The sterilized formulations were characterized as described above.

2.2.5. Protein integrity

2.2.5.1. Western blot analysis. GDNF samples were obtained by extraction from microspheres following the same protocol used to determine the encapsulation efficiency. Non-reducing and adding SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed onto 15% polyacrylamide gels. After separation, protein samples were transferred onto nitrocellulose membranes. Western blot was performed by sitting the membrane with the blocking solution: 5% non-fat dry milk in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h, followed by the incubation with anti-GDNF antibody 1:100 (R&D Systems) in blocking buffer overnight at 4 °C. After washing with TBST, the blot was incubated in blocking solution with the secondary antibody anti-goat 1:5000 (Santa Cruz Biotechnology). Detection was achieved using the ECL Western Blotting Detection Kit (GE Healthcare). Unless specified, all incubations were performed at room temperature.

2.2.5.2. In vitro bioassays. Retinal cell isolation and survival assay: Mouse retinal cells were isolated as described previously (Jiang et al., 2010). Briefly, retinas from postnatal day 10 B6 mice were disinfected free from the posterior eyecup. Pooled retinal tissues were finely minced in Mg2+ /Ca2+-free Hanks' balanced salt solution (HBSS) and dissociated by incubating for 20 min at 37 °C in HBSS containing 0.1% collagenase. The supernatant containing liberated cells was centrifuged at 233 × g for 5 min at room temperature and the pellet was resuspended and seeded into 16-well chamber slides in neurobasal medium supplemented with B-27 supplement and N-2 supplement. Once isolated, 5 × 103 retinal cells were exposed to various conditioned media released from different GDNF loaded microspheres prepared by the three different methods (W/O/W, S/O/W 1, and S/O/W 2) in the three different conditions [non sterilized (NS), sterilized at room temperature (SRT), and sterilized at low temperature (SLT)]. In order to compare biological activity of GDNF from the different microspheres, aliquots of the same concentration from the release study according to ELISA quantification were chosen for each condition (NS, SRT, and SLT). Three different GDNF amounts were tested: 250 pg for non sterilized formulations (9 weeks of the release assay for W/O/W, 11 weeks for S/O/W 1, and 10 weeks for S/O/W 2), 70 pg for sterilized microspheres (with dry ice) (11 weeks of the release study for W/O/W, 10 weeks for S/O/W 1, and 9 weeks for S/O/W 2), and 5 pg for sterilized microspheres (at room conditions) (17 weeks for W/O/W, 16 weeks for S/O/W 1, and 13 weeks for S/O/W 2). At 40 h post plating, cultures were fixed and analyzed via tunel labeling to determine the percentage of cell death.

2.3. Statistical analysis

The data were expressed as means ± SD. Data between groups were compared using an analysis of variance (one-way ANOVA). Results were taken as significantly different at P < 0.05.

3. Results

3.1. Microsphere characterization

The microencapsulation methods used in this work are described in Scheme 1. The different techniques led to a high production yield in all cases (72.4 ± 7.0% for W/O/W, 79.7 ± 4.4%...
Scheme 1. Different techniques employed to encapsulate GDNF in PLGA microspheres.

Fig. 1. SEM images of microspheres (non-irradiated and irradiated at low or room temperature) generated with the following three methods: W/O/W (A, D, and G), S/O/W 1 (B, E, and H), and S/O/W 2 (C, F, and I). An example of the particle size distribution has been included for each formulation.
for S/O/W 1, and 80.2 ± 3.9% for S/O/W 2). Scanning electron microscopy showed spherical particles with different aspects in the three formulations (Fig. 1A–C). Microspheres prepared by the W/O/W technique, possessed visible large pores on the surface. On the contrary, the S/O/W 1 method produced microparticles with smooth surfaces and absence of pores. When Vit E was included in the inner O-phase (S/O/W 2), microspheres remained spherical and a high number of small pores were observed on the surface. The mean particle size values obtained for the three formulations ranged from 19 to 26 μm (Table 1), which is suitable for administration as suspension through standard injection needles (27–34 G) (Herrero-Vanrell and Rejo, 2001).

3.2. Encapsulation efficiency, in vitro release of GDNF and degradation of microspheres

Table 1 summarizes the GDNF loading data of the particles elaborated by the different emulsification techniques.

The inclusion of the neurotrophic factor GDNF in its molecular state (dissolved in the inner phase of the first emulsion) (W/O/W) resulted in higher encapsulation efficiency (30.0 ± 0.9%) when compared to that obtained when the protein was included as a solid in S/O/W 1 (23.4 ± 0.5%). The inclusion of Vit E in the inner phase of the S/O/W 2 preparation increased the protein entrapment rendering similar values to the double emulsion technique (30.4 ± 1.1%).

The release rate of GDNF from microspheres fabricated by the different techniques are shown in Fig. 2. The profile obtained showed the typical triphasic shape of PLGA systems for the three formulations. According to Table 2A, the initial phase was characterized by a burst effect (protein release during the first 24 h of the release assay) followed by a short rapid release period (1 week for W/O/W and 2 weeks for S/O/W 1 and S/O/W 2), and a second long period of slow release. In the third phase, the release rate was clearly higher for the W/O/W formulation in comparison to S/O/W 1 and S/O/W 2. The protein remaining after the third phase was progressively released until the end of the assay: 22.3 ± 1.3 pg GDNF/mg MS/day, 4.8 ± 0.4 pg GDNF/mg MS/day, and 14.4 ± 0.4 pg GDNF/mg MS/day for W/O/W, S/O/W 1, and S/O/W 2 respectively (Fig. 2).

The evolution of the PLGA molecular weight in microspheres was evaluated by GPC for the formulation S/O/W 2. Spheres were incubated in the release media following exactly the same protocol used for the protein release studies. A homogeneous Mw reduction of PLGA (initial Mw 35,076.8 ± 292.4 g/mol) occurred across each condition during the first 28 days, which lead to a critical molecular weight of 13,445.5 ± 566.4 g/mol (Fig. 3A).

3.3. Sterilized microspheres

Regardless of sterilization conditions or fabrication process, the morphology of microspheres was unmodified by the sterilization procedure (Fig. 1D–I). Moreover, no changes in the mean particle size were detected (P = 0.73, P = 0.66, and P = 0.79 for W/O/W, S/O/W 1, and S/O/W 2 respectively) (Table 1). The protein content in the microspheres after gamma irradiation was reduced in all formulations (Table 1). The percentage of protein contained into the microspheres sterilized in presence of dry ice was decreased by 68% for W/O/W and 40% for both S/O/W 1 and S/O/W 2, as determined by ELISA quantification. When irradiation was performed at room temperature, only 20%, 24%, and 27% of the initial content was lost.
loaded protein was quantified for W/O/W, S/O/W 1, and S/O/W 2 preparations respectively.

The effect of gamma irradiation on the GDNF release profile for each of the three different types of microspheres was investigated (Fig. 2 and Table 2B and C). The biotechnological product released from microspheres after sterilization (at room and low temperature) maintained the triphasic profile. Although no changes in the periods of time of each phase were identified, a reduction in the GDNF release rates was observed for W/O/W and S/O/W 1 microspheres during the first phase of the release assay. This was not the case of the S/O/W 2 formulation that released the same amount of GDNF during the first 14th days, both before and after sterilization when the samples were protected with dry ice (Table 2). Afterwards, significantly lower amounts of protein, compared with non sterilized microspheres, were progressively released until the end of the assay (133 days) for all microsphere batches: 5.7 ± 0.6, 3.2 ± 0.1, and 2.1 ± 0.0 pg GDNF/mg MS/day for W/O/W, S/O/W 1, and S/O/W 2 formulations sterilized at low temperature and 1.7 ± 1.1, 1.4 ± 0.1, and 1.0 ± 0.7 pg GDNF/mg MS/day for W/O/W, S/O/W 1, and S/O/W 2 formulations sterilized at room temperature.

When the PLGA molecular weight from microspheres prepared according to S/O/W 2 method was quantified by GPC after sterilization, a decrease from 35,076.8 ± 292.4 g/mol to 28,441.0 ± 279.3 g/mol was observed. The reduction in PLGA Mw was similar for microspheres sterilized both at room temperature and with dry ice (Fig. 3B and C). The evolution of the microsphere morphology at different time points of the release assay (0 h, 1 h, 4, 7, and 12 weeks) is shown in Fig. 4. As it can be seen, microspheres significantly changed their initial shape at 6 weeks. Subsequently, a shapeless mass of polymer was observed at 8 weeks of the release study.

3.4. Western blot analysis

Western blot analysis was carried out to evaluate the structural integrity of encapsulated protein before and after sterilization process (in presence or absence of dry ice) for W/O/W and S/O/W 2 microspheres. In non-reducing conditions, bands for the native dimer (25–37 kDa) appeared for samples obtained from both W/O/W and S/O/W 2 formulations. In both cases, additional bands of approximately 15–20 kDa were also detected, demonstrating
that the quaternary structure of this labile protein was modified (Fig. 4).

3.5. *In vitro* bioassays

Bioactivity assays were performed to determine the biological activity of the protein after microencapsulation, sterilization, and release. To test, mouse retinal cells were exposed to GDNF released from microspheres generated by each of the different techniques and sterilization conditions (non sterilized, sterilized at low temperature, and sterilized at room temperature). As shown in Fig. 5, when comparing the three methods employed (W/O/W, S/O/W 1, and S/O/W 2), significantly less retinal cell death (*P*<0.001) was always observed at 40 h post-plating when cells were cultured in the presence of GDNF released from microspheres fabricated by the S/O/W 2 method. Biological activity of GDNF at 250 pg (detected by ELISA) from non sterilized microspheres fabricated by the S/O/W 2 method resulted in a lower retinal cell death (8.2%) compared with S/O/W 1 (15.9%) and W/O/W (20.4%). Similarly, the retinal cell survival detected using GDNF released from sterilized microspheres, protected and non protected with dry ice, (70 pg and 5 pg GDNF determined by ELISA, respectively) was significantly higher (*P*<0.001) for the S/O/W 2 technique when compared to the W/O/W and S/O/W 1 methods. The percentage of cell death was resulted in 28.4% for W/O/W, 17.7% for S/O/W 1, and 14.1% for S/O/W 2 in microspheres sterilized at low temperature and 31.3% for W/O/W, 24.3% for S/O/W 1, and 19.5% for S/O/W 2 in microspheres sterilized at room temperature (Fig. 5).

4. Discussion

The encapsulation of neurotrophic factors in biodegradable PLGA microspheres provides a desirable therapeutic approach for the treatment of chronic neurodegenerative diseases. GDNF loaded PLGA microspheres have been already prepared and tested for the treatment of Parkinson's disease (Garbayo et al., 2008) or glaucomatous optic neuropathy (Checa-Casalengua et al., 2011). The manufacturing of these formulations for clinical use requires to either produced under aseptic conditions or terminally sterilization. The goal of this study was to evaluate whether the use of the active protein in its solid state combined with the inclusion of antioxidants would be considered as a useful strategy to preserve the bioactivity of neurotrophic factors such as GDNF after microencapsulation in PLGA microspheres and subsequent sterilization by gamma irradiation. The technological approaches can be extended to other biotechnological products encapsulated in PLGA microspheres.
Fig. 5. Analysis of GDNF function post-microsphere degradation. (A–I) Tunel assay performed on retinal cells culture in presence of released GDNF from microspheres generated by W/O/W (A, D, and G), S/O/W 1 (B, E, and H) and S/O/W 2 (C, F, and I) in each of the different conditions: non-sterilized microspheres (A–C), sterilized microspheres (with dry ice) (D–F), and sterilized microspheres (at room temperature) (G–I). (J) Tunel assay performed on retinal cells culture in presence of BSS. The amounts of GDNF cultured were 250 pg (A–C), 70 pg (D–F), and 5 pg (G–I) according to ELISA quantification (***P < 0.001).
Biological activity of active agents is essential and it must be assured in all steps of the microencapsulation process as well as after sterilization in the final formulation. As the pharmacological activity of the protein can be substantially altered due to the manufacturing conditions, the protection of the active agent employing technological strategies is critical to avoid immunogenicity complications (Jiskoot et al., 2012). In this context, previous studies have demonstrated that the use of the protein in its solid state can reduce non-desired denaturation and the aggregation phenomena observed after its dissolution and posterior precipitation (Andreas et al., 2011; Perez et al., 2002).

Three solvent evaporation methods, W/O/W, S/O/W 1, and S/O/W 2 (including Vit E), were applied to prepare PLGA microspheres containing GDNF. In the W/O/W method, the neurotrophic factor was first dissolved in the aqueous internal phase (W1; PBS, pH 7.4). On the contrary, in the S/O/W methods, GDNF was encapsulated in its solid state, employing very mild conditions (short time, low power, and low temperature) to reduce cavitation stress during the formation of a homogeneous suspension of the protein (Scheme 1). GDNF was dispersed as solid in a volume of vitamin E in the inner phase of the emulsion (S/O/W 2 technique) resulting both compounds entrapped in the microspheres.

As expected, the different microencapsulation techniques employed had a significant effect on GDNF entrapment, as well as release kinetics. The W/O/W technique has been described as the most efficient means of loading PLGA microspheres with water-soluble proteins (Sinha and Trehan, 2003; Andreas et al., 2011; Lagarce et al., 2006; Wang et al., 2004). In our conditions, the inclusion of GDNF in the aqueous solution (W/O/W) led to an encapsulation efficiency of around 30%. Although this method allows protein loading up to 90%, the optimization of the procedure was beyond the scope of these experiments. Regarding the content of GDNF in the PLGA microspheres prepared with the solid protein, it is interesting to note that the inclusion of Vit E in the inner phase of the emulsion (S/O/W 2 method) had a beneficial effect increasing the amount of protein encapsulated compared to the S/O/W 1 technique. As shown in a previous report, the higher entrapment observed could be related to the disposition of the fatty additive close to the particle surface (Checa-Casalengua et al., 2011), which might reduce the diffusion of the protein to the external phase during particle hardening. The cumulative in vitro release profiles of GDNF from microspheres fabricated by the diverse techniques showed the typical triphasic shape characteristic of PLGA systems.

Injectable drug delivery systems loaded with biological products require effective sterilization. Gamma irradiation is one of the most widely used and effective sterilization procedures available (Fernandez-Carballedo et al., 2006; Herrera-Vanrell and Rejofio, 2001; Fried and Schlap, 2006; Putli and Vavia, 2009). However, ionizing radiation can have effects on the biodegradable polyesters, mainly by the formation of reactive radicals that might compromise the active substances included in these systems (Faisant et al., 2003; Mohr et al., 1999). Therefore, one approach to optimize the irradiation process could be the addition of substances able to protect the protein from the reactive oxygen species (ROS) produced as by-products of irradiation (Zbikowska et al., 2006). In this sense, the use of an oxidation scavenger (ascorbate) has previously been shown to prevent radical induced degradation, favoring the stability of the proteins in gamma sterilized plasma samples (Zbikowska et al., 2006). To our knowledge, this is the first study in which the microencapsulation of an active protein (GDNF) in its molecular or in its solid state are compared in terms of biological activity after gamma sterilization as well as the beneficial effects of an antioxidant (Vit E) included in the formulation as a potential protection agent against bioactivity loss of the microencapsulated protein.

The effect of gamma-irradiation on GDNF release profile from microspheres prepared by the different methods led to a higher initial burst for the three formulations. These results are in accordance with a previous work in which a higher burst effect release of the peptide rhIGF-1 (insulin like growth factor-I) was observed for irradiated microspheres (Carrascosa et al., 2003). Other authors also observed a faster release of the microencapsulated peptide (SP66) after irradiation (Igarta et al., 2008). In the present work, comparing the three methods employed to fabricate the microspheres, the release rate of the protein after sterilization was less modified using the S/O/W 2 technique. During the first period of the release assay (1–14 days), the amount of GDNF released from the S/O/W 2 microspheres remained practically unchanged after γ-sterilization (protected with dry ice), with values of 274.6 ± 36.9 pg GDNF/mg MS/day for non sterilized microspheres and 246.3 ± 76.4 pg GDNF/mg MS/day for microspheres sterilized at low temperature. In contrast, a significant reduction (more than two fold) was observed for the GDNF released from microspheres prepared according to W/O/W and S/O/W 1 techniques for the first 7 and 14 days of the release assay, respectively.

Since the successful development of injectable drug delivery systems containing proteins requires the protection of the protein biological function throughout microencapsulation and sterilization, the structural integrity and biological activity of GDNF into the microspheres were analyzed. Electrophoresis studies demonstrated the existence of bands corresponding to native protein (25–37 kDa) and additional bands for monomers (15–20 kDa), indicating structural changes in the protein. However, we cannot assure that the modifications observed were consequence of microencapsulation and sterilization because GDNF was extracted from the microspheres with the help of an organic solvent (methylene chloride). It is well known that this condition often perturb protein structure, inducing protein aggregates and/or accelerate chemical degradation (Jiskoot et al., 2012).

The most relevant results of the present study are related to functional activity of GDNF in their potential target cells (retinal cells) from the different formulations and conditions. In all cases, the S/O/W 2 formulation seemed to offer better perspectives, according to the evaluation of retinal cell survival of GDNF released from the three formulations. In bioassays, equal amounts of GDNF (quantified by ELISA) released from each formulation in each condition (non sterilized, sterilized at low temperature, and sterilized at room temperature) were compared. Three different amounts of the biotechnological product (250 pg for NS, 70 pg for SLT, and 5 pg for SRT) were tested. In all cases, the cell survival resulted always higher for the GDNF released from the S/O/W 2 microspheres. However, considering that ELISA can determine both monomers and dimers and only the latest are active, the higher bioactivity observed for the protein released from all microspheres prepared by the S/O/W 2 technique are indicative to a more preservation of the native structure due to the use of protein in its solid state and the inclusion of antioxidants.

5. Conclusions

Although none of the systems proposed achieved with the goal of totally retain the structural integrity of GDNF, the use of solid proteins in combination with antioxidants, can be considered an appropriated start-point to develop formulations able to preserve the biological activity of microencapsulated biotechnological products after sterilization by gamma irradiation at low temperature (−78 °C).

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