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

P. Checa-Casalengua[a,1], C. Jiang[b,1], I. Bravo-Osuna[a], B.A. Tucker[c], I.T. Molina-Martínez[a], M.J. Young[b], R. Herrero-Vanrell[a,*]

[a] Dep. of Pharmacy and Pharmaceutical Technology, School of Pharmacy, Avd. Complutense s/n, Complutense University, Madrid 28040, Spain
[b] Schepens Eye Research Institute, Dep. of Ophthalmology, Harvard Medical School, Harvard University, 20 Staniford Street, Boston, MA 02114, USA
[c] Institute for Vision Research, Dep. of Ophthalmology, Carver College of Medicine, University of Iowa, 4187 MERF, Iowa City, IA 52242, USA

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ABSTRACT

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.

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

* Corresponding author. Tel.: +34 913 941 739; fax: +34 913 941 736.
E-mail address: rociohv@farm.ucm.es (R. Herrero-Vanrell).
[1] These authors have collaborated in the same extent.

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et al., 1995; Montanari et al., 1998; Bittner et al., 1999; Faisant et al., 2002; Martinez-Sancho et al., 2004; Fernandez-Carballido 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 peptides and proteins (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 (Martinez-Sancho et al., 2004; Fernandez-Carballido 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 (Martinez-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 dimer 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 inmunosorbant 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 Mw 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-Tocoferol 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 (CH_2Cl_2) (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/CH_2Cl_2 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