Microencapsulation of recombinant interferon α -2b into poly (D,L-lactide-co-glycolide) microspheres

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ABSTRACT

Interferon- $\alpha 2b$ (IFN- $\alpha 2b$) produced in Cuba by the Center for Genetic Engineering and Biotechnology was encapsulated into poly (D,L-lactide-co-glycolide) (PLGA) microspheres using the double emulsion-solvent evaporation method. Resulting microspheres showed smooth surfaces containing randomly distributed pores, a mean diameter of 28.1 ± 0.4 µm (using 14,000 rpm for the second emulsification step), PLGA recovery of 86 ± 1%, loading between 0.41 and 1.23% for interferon at 5-20 mg/mL in the inner aqueous phase, with the corresponding encapsulation efficiency of 79 and 56%. The encapsulated interferon was extracted by both passive diffusion and solvent extraction techniques and further characterized. No changes were detected in the physico-chemical and biological characteristics of the IFN- α 2b recovered by diffusion-controlled release for 24 h at 37 °C. In contrast, the solvent-extracted fraction showed 43 ± 4% of immunorecognized IFN- α 2b and 61% of its initial antiviral activity (1.7 x 10⁸ IU/mg). These instabilities were due to the encapsulation method, and not to spontaneous IFN- α 2b modifications. Consequently, IFN- α 2b showed potential for encapsulation in PLGA microspheres for controlled release.

Keywords: poly (lactide-co-glycolide), microspheres, interferon, controlled release, protein delivery

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RESUMEN

Microencapsulación de interferón α -2b en microesferas de poli (D,L-lactida-co-glicolida). En este trabajo se encapsuló interferón- α 2b (IFN- α 2b), producido en Cuba por el Centro de Ingeniería Genética y Biotecnología, en microesferas de poli (D,L-lactida-co-glicolida) (PLGA) por medio del método de doble emulsión-evaporación del disolvente. Las microesferas resultantes mostraron superficies regulares con poros distribuidos aleatoriamente, un diámetro promedio de 28.1 ± 0.4 µm (al emplear 14 000 rpm para obtener la segunda emulsión), un recobrado de PLGA de 86 ± 1%, cargas entre 0.41 y 1.23% al utilizar interferón a 5-20 mg/mL en la fase acuosa interna, con eficiencias de encapsulación correspondientes entre 79 y 56%. El interferón encapsulado fue extraído de las microesferas para su caracterización, mediante difusión pasiva o por la acción de disolventes. No se detectaron cambios en las características físico-químicas y biológicas del IFN- α 2b liberado por difusión durante 24 h a 37 °C. Sin embargo, la fracción de IFN- α 2b extraída por medio de disolventes mostró un inmuno-reconocimiento de 43 ± 4% y 61% de su actividad antiviral inicial (1.7 x 10⁸ IU/mg). Estos cambios se debieron al método de encapsulación y no a modificaciones espontáneas del IFN- α 2b. En consecuencia, el IFN- α 2b puede, potencialmente, ser encapsulado en microesferas de PLGA para su aplicación en liberación controlada.

Palabras clave: poli (lactida-co-glicolida), microesferas, interferón, liberación controlada, administración de proteínas

Introduction

The discovery and clinical investigations of interferons (IFNs, *e.g.*, IFN- α , IFN- β , IFN- γ , etc.) have remarkably contributed to the development of both modern biotechnology and medicine associated to the production and therapeutic use of proteins. A variety of past and current clinical trials have found their support in the immuno-modulatory, antiproliferative and antiviral properties of IFNs[1]. Consequently, there are some interferon-based products already approved for treating several types of cancer and multiple viral diseases ([1] and references therein).

At present, IFNs can mainly be obtained from either natural sources [2, 3] or genetically modified organisms [4]. However, the IFNs of recombinant origin are favored for their production by the biotechnological industry, because of their homogeneity and greater productive yields. In Cuba, the obtainment and development of human interferon alpha 2b (IFN- α 2b) from recombinant strains of *Escherichia coli* started in 1987. The effectiveness of this cytokine for the treatment of both oncological and viral pathologies has been widely investigated and demonstrated [5-7].

The Cuban IFN- α 2b is currently produced with the proper physical-chemical and biological quality for its use as the active ingredient of several biopharmaceutical dosage forms [8, 9]. The quality of IFN- α 2b presentations (*e.g.*, freeze-dried Heberon Alfa R[®] from Heber Biotec SA Havana, Cuba) has also been confirmed in comparison studies using equivalent formulations from other commercial sources (*e.g.*, from Schering-Plough Corp, Hoffmann-La Roche, INMUNO SA, Instituto SIDUS SA) [8]. Recently a new liquid, albumin-free parenteral IFN- α 2b formulation has been developed and licensed for use in humans [10, 11]. The long-term (at least 30 months) stability of this formulation, when it is

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3. Lemieux R, Neron S, Proulx C, inventors; Hema-Quebec (CA); Canadian Blood (CA). Method of producing human IFNalpha using sendai virus-infected hematopoietic stem cells. US Patent 6,472, 208. 2002. stored at 4 °C, emphasizes the quality of the IFN- $\alpha 2b$ [10].

As in other proteins that are administered using fast delivery formulations, the Cuban IFN- α 2b exhibits a relatively short elimination half-life (5.87 h) [11]. Therefore, a high frequency of IFN- α 2b administration is needed, to achieve effectiveness in most interferon-treated diseases [5, 12]. To attenuate this problem, the conjugation of IFN- α 2b to a branched polyethyle-ne glycol (40 000) is currently being developed [13].

Microencapsulation using polymeric biodegradable matrices is one of the technologies that could potentially (*i*) reduce the administration frequency of the Cuban IFN- α 2b, (*ii*) maintain appropriate IFN levels in the blood for an adequate period of time, and hence (*iii*) improve the compliance of patients during the treatment with IFN- α 2b [14, 15]. Derived microsphere-based formulations would also lead to a greater therapeutic benefit due to the elimination of IFN- α 2b fluctuations in serum.

Consequently, the aim here was to explore the encapsulation of IFN-a2b into poly (D,L-lactide-coglycolide) (PLGA) microspheres. The resulting IFN- α 2b-loaded microspheres, which were obtained using the double emulsion-solvent evaporation method [16], were characterized by: 1) scanning electron microscopy for morphology; 2) laser diffractometry for particle size distribution and average diameter; 3) microparticle solubilization with NaOH followed by microBCA protein assay for IFN-a2b loading and encapsulation efficiency; and 4) gel permeation chromatography for PLGA molecular mass determination (before and after passive diffusion-controlled release of IFN- α 2b from the microspheres). The effect of key experimental parameters were also evaluated. such as the concentration of IFN- α 2b in the inner aqueous phase from the primary emulsion and the stirring speed of the second emulsification step on the characteristics of the microspheres. We then extracted IFN- α 2b from the microspheres by means of both passive diffusion and solvent extraction for further characterization (*i.e.*, integrity determination) using i) slab-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the presence of IFN-α2b aggregates; *ii*) reversed-phase high-performance liquid chromatography (RP-HPLC); iii) enzyme-linked immunosorbent assay (ELISA) for measuring the immunorecognition of IFN- α 2b by anti-IFN- α 2b monoclonal antibodies (MAb); and *iv*) in vitro bioassay for evaluating the effect of the encapsulation process on IFN- α 2b antiviral activity.

The potential of the Cuban IFN- α 2b to be used for PLGA microsphere-based controlled delivery is demonstrated.

Materials and methods

Materials and reagents

Clinical grade recombinant IFN- α 2b, anti-recombinant IFN- α 2b CBIFNA 2.3 monoclonal antibody and antirecombinant IFN- α 2b CBIFNA 2.4-HRP conjugate were commercial products supplied by the Center for Genetic Engineering and Biotechnology (Havana, Cuba). Poly (D,L-lactide-co-glycolide) copolymer (Lactel BP-0100, 50:50 D,L-lactide:glycolide molar ratio) and polyvinyl alcohol 30 000-70 000 were from Sigma (St. Louis, Missouri, USA). All other reagents were obtained from Merck (Darmstadt, Germany).

Preparation of microspheres

Microspheres containing IFN-a2b were prepared using the double emulsion-solvent evaporation method according to Cohen et al. [16] with some modifications. Briefly, 100 µL of the internal aqueous phase (10 mM sodium phosphate, 145 mM NaCl, pH 7.2 (PBS)) containing IFN-a2b (5-20 mg/ml) were added to 1 mL of the polymer solution (10% PLGA in dichloromethane). The mixture was sonicated for 30 s on ice using an IKASONIC U 200 S ultrasonic homogenizer with a sonotrode #2 (IKA Labortechnik, Germany). The resulting primary water-in-oil (w/o) emulsion was added to 40 mL of the external aqueous solution (1% w/v polyvinyl alcohol). A second emulsion (w/o/w) was obtained by stirring the mixture with a high-speed homogenizer Ultraturrax T8 (IKA Labortechnik, Germany) for 2 min on ice. The double emulsion was poured into 140 mL of the polyvinyl alcohol solution and stirred, at 300 rpm for 30 min, with a homogenizer (IKA Labortechnik, Germany) to extract and evaporate the dichloromethane. Finally, formed microspheres were collected by filtration through a 0.45 µm cellulose nitrate membrane (Sartorius GmbH, Germany), washed 5 times with distilled water, dried in a freezedryer (Edwards, UK) during 16 h (-40 °C, 10⁻³mbar). Microparticles were stored at 4 °C. Blank microspheres were prepared with PBS instead of the IFN-α2b solution. Everytime this process was carried out, the recovery of microspheres was calculated with respect to the initial mass of polymer. This parameter was calculated as the ratio of final to initial polymeric material mass and expressed in percentage. For the calculation, the amount of PLGA contained in the organic phase to form the primary w/o emulsion was taken as the initial polymer mass. The difference between the mass of the vial containing the dried microspheres and the empty vial was considered as the final polymeric material mass.

Characterization of microspheres

Morphology

Dried microspheres containing IFN- α 2b were deposited on the conductive surface of a double slide carbon adhesive tape, previously fixed to a sample support disc. They were then coated with platinum for 2 min in an ion sputter-coater JFC-1100 (Jeol, Japan) with a direct current of 15 mA (1.2 kV). Morphological characteristics of the microspheres were examined by imaging the coated samples with a Philips XL-20 scanning electron microscope (Philips Electron Optics, Eindhoven, Netherlands) operated at 5 kV.

Particle size determination

Twenty mg of microspheres were suspended in $100 \,\mu\text{L}$ of distilled water. The resulting suspension was sonicated for 5 s to disaggregate the microspheres. Then $20 \,\mu\text{L}$ of the preparation were loaded into the sample cell of a laser diffraction particle size analyzer Coulter LS 230 (Coulter, USA). The size distribution of the

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 Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. Controlled delivery systems for proteins based on poly (lactic/ glycolic acid) microspheres. Pharm Res 1991;8:713-20. microsphere samples was determined according to a programmed model, based on the Fraunhofer theory of light scattering [17].

Interferon-loading and encapsulation efficiency

Ten milligrams of microspheres were hydrolyzed under moderate stirring with 1N NaOH (0.5 mL) for 16 h at room temperature (25 °C). The resulting solution was neutralized with 1N HCl and IFN- α 2b concentration was estimated with the bicinchoninic acid method using the microBCA protein assay reagent kit (Pierce, Rockford, IL, USA). The IFN- α 2b encapsulation efficiency (EE) was calculated as the ratio of experimental to theoretical IFN loading, and expressed as percentage. The experimental loading level of IFN- α 2b was determined as the mass of IFN- α 2b recovered from the microspheres divided by the mass of the hydrolized microspheres, and expressed as percentage (w/w). The theoretical IFN loading parameter was defined as the initial mass of IFN- α 2b introduced in the process divided by the initial mass of PLGA. We assumed that the mass of IFN- α 2b is very small compared to that of PLGA.

Integrity of the encapsulated IFN- α 2b

Two different types of experiments were carried out for determining the integrity of the IFN- α 2b within the microspheres: passive diffusion and solventassisted extraction.

Passive diffusion of IFN- α 2b

Fifty milligrams of the microspheres containing IFN- α 2b were suspended in 1 mL of the release medium (PBS, pH 7.2, containing Tween 80 (0.001%) and sodium azide (0.1%)). The suspension was gently stirred in a Roto-Shake Genie shaker (Scientific Industries Inc., Bohemia, NY, USA) and incubated at 37 °C. At specified time intervals (0.33 (8h), 1, 7, 14, 21 and 28 days), samples were centrifuged for 5 min at 5 000 rpm in a Hettich centrifuge (Tuttlingen, Germany) and 50 µL of the supernatant were with drawn. The volume extracted was replaced with an equal volume of the fresh release medium. The concentration of IFN- α 2b in each sample was assessed with the microBCA assay and the IFN-a2b released was calculated with respect to the total mass of IFN- α 2b contained in the suspended microspheres. Since the diffusioncontrolled IFN- α 2b released after the first day was negligible, the only fraction analyzed was that corresponding to the first 24 h. The following analytical techniques were used: RP-HPLC, SDS-PAGE, ELISA and biological antiviral activity. The results were compared with those from the non-encapsulated IFN- α 2b sample, which was incubated under the same conditions as used to incubate the microspheres.

Also, the microspheres before and after incubation for passive diffusion were examined by scanning electron microscopy and gel permeation chromatography, to evaluate both particle erosion and polymer degradation.

Extraction of IFN- α 2b from microspheres

IFN- α 2b was extracted from the microspheres with the aid of the solvent system (a mixture of dichlorometha-

ne and acetone (1:3 (v/v)) described by Johnson *et al.* [18]. Twenty milligrams of microspheres containing IFN- α 2b were mixed with 2 mL of the solvent, vortexed for 1 min in a MS2 minishaker (IKA Works Inc., Wilmington, NC, USA), and then gently agitated for another 20 min in a Roto-Shake Genie shaker (Scientific Industries Inc., Bohemia, NY, USA). The resulting suspension was centrifuged at 5 000 rpm in a Hettich centrifuge (Tuttlingen, Germany) for 5 min. After the supernatant was discarded, the pellet was resuspended in 2 mL of the solvent system and stirred for 10 min. The sample was centrifuged again and the pelleted IFN- α 2b was recovered and dried in a freeze-dryer (Edwards, UK) during 6 h (-40 °C, 10⁻³mbar). Then, it was dissolved in 100 µL of PBS, pH 7.2. Samples were analyzed by these same analytical techniques. Appropriate controls were prepared, processed and analyzed under the same experimental conditions. The IFN- α 2b mass was determined in each sample using the microBCA assay. Protein recovery was calculated in relation to the IFN-a2b mass contained in the microspheres or the IFN-a2b mass used to prepared the other samples (C, D, E and F in table 1).

Analytical techniques

Optical microscopy analysis

Two mg of the microspheres containing IFN- α 2b were suspended in 20 µL of distilled water. The suspension was placed on a micro slide, with a glass capillary, and dried in an oven (Memmert, Germany) at 60 °C for 1 h. The microspheres were observed using a Fluovert optical microscope (Leitz, Wetzlar, Germany) and a Wild M8 stereomicroscope (Leica, Germany).

SDS-PAGE

The IFN- α 2b samples (recovered by passive diffusioncontrolled release or solvent-assisted extraction) were diluted 1:5 (v/v) in 625 mM Tris-HCl buffer (pH 6.8) containing 5% sodium dodecyl sulphate, 50% (v/v) glycerol and 0.025% bromophenol blue. Then, they were run in a 12.5% acrylamide gel, as described by Laemmli [19]. The electrophoresed gels were stained for protein with Coomassie brilliant blue, and analyzed densitometrically with a ScanJet 4c/T scanner (Hewlett Packard, Palo Alto, CA, USA) and Molecular Analyst software (Bio-Rad Laboratory, Hercules, CA, USA).

RP-HPLC

RP-HPLC analysis was performed on a Vydac (Hesperia, CA, USA) wide pore octyl C8 column (5 μm, 250 mm x 4.6 mm). The elution was made, in 55 min, using a gradient from 15 to 65% of A in B (A: 0.05% trifluoroacetic acid in acetonitrile; B: 0.1% trifluoroacetic acid in water). The flow rate was 0.8 mL/min. Detection of IFN- α 2b was performed at 226 nm with an automatic data processing, by means of D-7 000 Multi HSM software (Merck, Darmstadt, Germany).

Gel permeation chromatography

A high-performance liquid chromatography system (LaChrom) provided with a refractive index detector was utilized (Merck, Darmstadt, Germany). The polymeric matrix dissolved in tetrahydrofuran were eluted with the same solvent at 1 mL/min through a 17. Freitas S, Rudolf B, Merkle HP, Gander B. Flow-through ultrasonic emulsification combined with static micromixing for aseptic production of microspheres by solvent extraction. Eur J Pharm Biopharm 2005;61:181-7.

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TSK-GEL GMHHR-N column (7.8 mm x 30 cm; 5 µm) from TOSOH BIOSEP, Japan. Molecular weight was calculated relative to polystyrene standards (TOSOH BIOSEP, Japan; MW range: 1.050 - 96.400 g/mol). The results were corrected according to the Mark-Houwink equation (Mark-Houwink coefficients in THF: $K_{PLGA50:50} = 1.07 \times 10^{-2} \text{ mL/g}$, $a_{PLGA50:50} = 0.76$; $K_{FS} = 1.41 \times 10^{-2} \text{ mL/g}$, $a_{PS} = 0.74$) [20].

ELISA

This analysis was made as described by Santana et al. [21]. Each well of the microtiter plate was coated with 1.5 µg of anti-recombinant IFN-a2b CBIFNA 2.3 MAb in 0.1 mL coating buffer (0.05 M Na₂CO₃, 0.05 M NaHCO₃, pH 9.6) and incubated for 3 h at 37 °C. The coated plate was washed twice with washing solution (PBS, pH 7.2, containing 0.05% (v/v) polysorbate 20). Then, 100 µL-diluted samples were added per well in the assay buffer (PBS containing 0.5% (w/v) skim milk). After incubation for 30 min at 37 °C, the plate was washed five times with PBS/ polysorbate 20. Then 70 µL of the conjugated second antibody (anti-IFN-a2b CBIFNA 2.4 MAb) labeled with horseradish peroxidase, diluted 1:10 000 (v/v) in the assay buffer, were added and incubated at 23 °C for 1 h. The plate was washed eight times with PBS/ polysorbate 20, and then incubated for 15 min at 23 °C with 100 µL of the substrate solution. The substrate solution contained 0.1 M Na₂HPO₄, 0.048 M citric acid, pH 5.5, 0.45 g o-phenylenediamine/L plus $0.3 \text{ g H}_2\text{O}_2/\text{L}$. The reaction was stopped by adding 50 µL of 2.5 M H₂SO₄. Absorbance was measured at 492 nm by using an ELISA-plate reader (SensIdent Scan; Merck, Darmstadt, Germany).

Antiviral activity assay

Antiviral activity of IFN-a2b was assayed by inhibition of the cytopathic effect produced by the Mengo virus with Hep-2 cells (ATCC No. CCL23) [22]. Samples of IFN- α 2b, serially diluted 1:2 (v/v) in Minimum Essential Medium containing 2% fetal calf serum and 40 µg/mL gentamicin, were mixed with cell monolayers in 96-well microtiter plates. The plates were then incubated at 37 °C for $2\hat{4}$ h, under $3\hat{\%}$ CO₂ and 95% humidity. After adding the virus (10^7 TCID), the plates were incubated until the cytopathic effect (90% cell lysis) was evident (approximately 18-20 h) in the virus control wells (without IFN- α 2b). The degree of cell destruction was measured by staining the remaining cells with crystal violet. A microplate reader (Tecnosuma, Havana, Cuba) was used to determine the cytopathic effect. The resulting data were transformed to a linear regression through a probit transformation with the ParLin 50 software (Center for Genetic Engineering and Biotechnology, Havana, Cuba), which is validated and registered. The unit of antiviral activity was defined as the reciprocal of the sample dilution that yields 50% protection of cells against the Mengo-induced cytopathic effect. The potency of each sample was expressed in IU, compared to a secondary reference calibrated against the 69/19 International World Health Organization IFN-α2b standard.

Results and discussion

More than 40 independent microsphere experimental batches (empty or loaded with IFN- α 2b) were manufactured with different aims. The manufacturing process involved five well-defined stages, as summarized in figure 1.

The drying step was done in about 18 h and the whole microencapsulation process took approximately 20 h. The polymer (PLGA) average recovery for the microsphere preparations was $86 \pm 1\%$ (n = 40). Soriano *et al.* previously obtained similar results (89.9 ± 0.6%) when they prepared 5 experimental batches of PLGA-microspheres containing bovine serum albumin [23]. Equally, Jung *et al.* have described yields as high as 86.8, 91.6 and 79.3% for three different microsphere preparations (PLGA 50:50, PLGA 75:25, polyoxy-ethylene/PLGA block co-polymer) that had in common the encapsulation of the tetanus toxoid and the use of the same manufacturing method (double emulsion-solvent evaporation) [24].

Morphology and particle size

Morphology

Figure 2 shows the morphological characteristics of the microparticles prepared. They are visualized as microspheres of smooth surfaces containing a few ran20. Schwarz K, Epple M. A detailed characterization of polyglycolide prepared by solid state polycondensation reaction. Macromol Chem Phys 1999;200:2221-9.

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Figure 1. Schematic diagram of the double emulsion-solvent evaporation method for obtaining the microspheres of PLGA loaded with IFN- α 2b. DCM - dichloromethane, PVA - polyvinyl alcohol.

domly distributed small pores. Although it was not verified here, these PLGA-type microspheres are known to have a very porous honeycomb like structure [25].

The microscopic analysis also revealed the presence of defects in some particles that were probably generated during microsphere formation. Possibly these defects were caused by the rapid extraction of the solvent (dichloromethane) from the microdroplets (microsphere precursors) of the second emulsification step in the manufacturing process (Figure 1). It is possible to reduce solvent extraction speed to minimize defects, and this can be achieved by adding an appropriate amount of dichloromethane to the external aqueous phase. We did not follow this approach because the efficiency of protein encapsulation would decrease, as previously described [26].

Effect of stirring speed on the particle size

The speed at which the second emulsification is made is one of most important factors affecting microsphere size [27]. Accordingly, figure 3 shows that increasing the stirring speed (from 8 000 to 20 000 rpm) reduced mean microsphere size. The same effect has been observed by others [27, 28].

Using optical microscopy (data not shown), we found that (i) stirring at less than 6 000 rpm the microparticles formed were not spherical and (ii) at 8 000 rpm, they were approximately spherical in shape. The microparticles manufactured at 8 000 rpm were spheres with a mean diameter of 60 µm, as determined using laser diffractometry (Figure 3). Nevertheless, minor microsphere populations with a diameter larger than 180 µm were also observed (Figure 3); this value limits the size for the particles to be included in parenteral formulations [29]. Figure 3 also shows that the microspheres obtained at higher speeds (14 000 or 20 000 rpm) had a smaller diameter (21 µm and 28 µm respectively), and that the generation of microparticle populations of larger sizes did not occur.

Consequently, we chose 14 000 rpm as the stirring speed during the secondary emulsification step in the subsequent experimental work. Five independent microsphere experimental batches containing IFN- α 2b, performed under the selected experimental conditions (volume of the internal aqueous phase, 100 µL; volume of the organic phase, 1 mL; volume of the external aqueous phase, 40 mL; stirring speed for the second emulsification step: 14 000 rpm), showed similar particle sizes (27.3, 27.3, 27.8, 28.1 and 29.7 µm) with a mean diameter of 28.1 ± 0.4 µm. This verified the excellent reproducibility of the process chosen.

Encapsulation efficiency and interferon loading

Encapsulation efficiency

This depends on the experimental conditions used to form the microspheres. The volume and the concentration of the protein in the inner aqueous phase, the polymer concentration in the organic phase, the polymer type, the times required for emulsification, as well as additives included in the different phases of microsphere assembly are the factors that most strongly affect encapsulation efficiency [30]. Some



Figure 2. Micrographs of PLGA microspheres loaded with IFN- α 2b. The microspheres were made with the double emulsion-solvent evaporation method, using 14 000 rpm for stirring the second emulsion. Particle size distribution is shown in (a), and surface morphology in (B, C, D). The defects in some particles are indicated with arrows (in C and D).

values described for this parameter are: 85.3-90.4% for the human growth hormone [31], 39-85% for lysozyme [32, 33], 45-48% for carbonic anhydrase [34], 100% for IFN- γ [35], 81-85% for insulin-like growth factor [36] and 55.8% for prolidase [37].

Using different IFN- α 2b concentrations (5, 10 and 20 mg/mL), diverse encapsulation efficiency values were obtained (Figure 4). This graphic also showed that this parameter was higher for the microsphere preparations made with lower IFN- α 2b concentration in the internal aqueous phase. A least-significant difference test revealed that the average value for the

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Figure 3. Effect of the stirring speed during the second emulsification step on the size of the microspheres.



Figure 4. Effect of the IFN- $\alpha 2b$ concentration in the internal aqueous phase on protein loading and encapsulation efficiency for the double emulsion-solvent evaporation-generated microspheres.

encapsulation efficiency (79%) using 5 mg/mL of the protein was significantly different (p < 0.01) from the average values obtained using 10 and 20 mg/mL, which were 59% and 56%, respectively. This tendency is in agreement with previous results where increasing the human serum albumin concentration negatively influenced encapsulation efficiency [38]. Considering that in some experiments the efficiency was over 80% (data not shown), we believe that the present procedure (Figure 1) might still be susceptible to improvement. Consequently, further experiments that might also help in achieving this goal should be considered, including the manipulation of such parameters as the volume and use of additives (e.g., carboxymethylcellulose, mannitol) in the inner aqueous phase [38]. In fact, PLGA microspheres loaded with either human serum albumin or nerve growth factor have been able to encapsulate these proteins improving from 5 to 77% in the initial works [38] to 95% or higher in later works [39, 40].

We also studied the effect of the inner aqueous phase volume on the IFN- α 2b encapsulation efficiency. The emulsification of 50 µL IFN- α 2b solution enabled us to encapsulate 66 ± 1% (n=5) of the initial IFN- α 2b. Using 100 µL of the same IFN- α 2b solution (20 mg IFN- α 2b/mL) the encapsulation efficiency decreased to 56 ± 2%. Similar observations using both human and bovine serum albumins have been previously described [38, 41].

Interferon loading

Different protein loading values, ranging between 0.34 and 1.63%, were obtained for the different manufactured microsphere experimental batches. The protein loading values corresponding to each IFN- α 2b concentration (5, 10, or 20 mg/mL) were found to be reproducible (data not shown). In contrast to the behavior of encapsulation efficiency, the average protein loading was higher when more concentrated IFN- α 2b solutions were used (0.65 ± 0.02% and 1.23 ± 0.05% for 10 and 20 mg of IFN- α 2b/ mL, respectively), as shown in figure 4.

These IFN- α 2b loading values were higher than those described by other authors who used similar protein concentrations. Genta *et al.* encapsulated prolidase using a 20 mg/mL protein solution, and the same polymer concentration (10%) and the ratio between the aqueous and organic phases (1:10) as used here [37]. They obtained a protein loading of 0.6%, whereas we reached an average of $1.23 \pm 0.05\%$. Using native or polyethylene glycol-conjugated epidermal growth factor, Kim *et al.* attained loadings of 0.18 and 0.21%, respectively [42]. They emulsified an inner aqueous phase containing 10 mg of protein per milliliter. Working with a similar IFN- α 2b concentration, we were able to reach an average of 0.65%. Since many experimental variables strongly influence the protein loading parameter, it is quite difficult to satisfactorily explain the differences between these results.

Integrity of the encapsulated IFN- α 2b

For a useful delivery system, microspheres should retain, to a great extent, the initial properties of encapsulated protein [15]. Interferon-recovery experiments might serve as an indicator to assess the effect of the present microencapsulation conditions on the cytokine. To this end, we first extracted IFN- α 2b from PLGA microspheres using two methods: long-term passive diffusion [35, 43] and solvent extraction [18, 33, 44].

IFN-α2b passive diffusion

Many authors have reported an incomplete release profile for several proteins. They have associated their results to: *i*) changes in the molecule such as the conversion into partially or completely aqueous-insoluble protein complexes, due to the stringency (*e.g.*, exposure to large air-water interfaces, mechanical agitation) during the encapsulation process, which can slow down or disable protein release towards the receiving fluid [31, 32, 45]; *ii*) nonspecific adsorption of the protein to polymer [31, 33] and *iii*) insufficient polymer degradation during the evaluation period [46].

In evaluating IFN- α 2b passive diffusion from PLGA microspheres during 28 days, we observed that this cytokine followed a two-stage release profile. Approximately 20% of the initial IFN- α 2b was released during the first day (burst release), where as only a small IFN- α 2b percentage (5%) was able to diffuse out during the rest of the period (27 days). The burst release effect may have likely been contributed by the fast diffusion of the IFN- α 2b located on the surface of the networks of inner pores and interchannels formed by evaporation of dichloromethane and water during the microsphere solidification process.

The second stage of the release profile is likely to correspond to the induction type-period before the start of PLGA polymer mass loss (bioerosion) due to the hydrolysis of ester bonds, and IFN- α 2b diffuses through both the existing and erosion-created channels [47]. In fact, scanning electron microscopy analysis revealed that the microspheres were almost unchanged before and after the release period (Figure 5). Only a slight erosion could be visualized in their surface. On the other hand, gel permeation chromatography (Figure 6) showed that the molecular mass of the degraded polymer remained high (Mr before release: 37 400; Mr after: 19 700). Thus, these high-molecular mass polymer fragments would likely prevent IFN-α2b exposition to the outer liquid phase, and consequently contribute greatly to the restricted diffusion of water27. Dinarvand R, Moghadam SH, Mohammadyari-Fard L, Atyabi E. Preparation of biodegradable microspheres and matrix devices containing naltrexone. AAPS Pharm Sci Tech 2003;4:E34.

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soluble IFN- α 2b species through the porous channels within the microspheres. The formation of new pores and channels, which would otherwise contribute to the passive diffusion-assisted IFN- α 2b release, was not favored either. Consequently, insufficient polymer degradation seems to be one of the reasons associated to the incomplete release of the IFN- α 2b from these microspheres. Other experiments to evaluate other probable causes (e.g., covalent (or not) aggregation, nonspecific adsorption) and gain further insight on the incomplete release of IFN- α 2b from the microspheres will be carried out later. In any event, our data are consistent with the biphasic profile seen for the release of many other peptides and proteins (e.g., growth factors and hormones, cytokines, antigens) from PLGA microsphere matrices [31, 32, 45, 46].

Our next step was to evaluate the properties of the IFN- α 2b recovered after the 24 h-passive diffusion.

RP-HPLC

This chromatography technique showed a main IFN- α 2b peak at a retention time (RT) of 36.77 min followed by another minor peak (RT = 38.31 min) corresponding to a more hydrophobic form of the IFN- α 2b (Figure 7A). No difference between this elution profile and the one corresponding to the free (control) IFN- α 2b, incubated under the same conditions as the microspheres, were found (Figure 7B). No traces of IFN- α 2b degradation products were detected, which would have indicated its chemical



Figure 6. Molecular mass distribution of the PLGA polymer in the microspheres before (dashed line) and after (continuous line) incubation in PBS (pH 7.2) at 37 °C. Samples were applied onto a TSK-GEL GMHHR-N (7.8 mm x 30 cm; 5 μ m) column, eluted with tetrahydrofurane at 1 mL/min and detected with a refractive index detector. Molecular mass was calculated relative to polystyrene standards.

modification (*e.g.*, oxidation, crosslinking disulfide scrambling, deamidation) within the microspheres.

SDS-PAGE

The dimeric fraction $(6 \pm 1\%)$ detected on slab PAGE from the passively eluted IFN- α 2b was similar to that of the control samples $(5 \pm 1\%)$ (Figure 8). Moreover, neither higher molecular mass species nor fragments of smaller molecular masses were observed in the released-IFN- α 2b samples (data not shown). This indicates that the burst release-associated IFN- α 2b population did not undergo (at least irreversible) aggregation or fragmentation during the encapsulation process (Figure 1). Using sodium dodecyl sulphatesize exclusion chromatography, Yang and Cleland [35], described that IFN- γ released from PLGA microspheres in a solution similar to the one used in this study, during the first day, had 98.5% of the dissociable fraction of IFN- γ .

Bioactivity

For PLGA microsphere-encapsulated IFN- γ , a high (87%) antiviral activity has been recovered after *in vitro* release for 24 h, as determined with a cytotoxic assay that uses human lung carcinoma (A549) cells [35]. In contrast, Sanchez and co-workers found that IFN- α released during the first day from PLGA microspheres containing poloxamer as the stabilizing agent did not produce significant levels of cytostasis in an *in vitro* antiproliferative test [43]. Likewise, Zhou and co-workers described that the IFN- α 2a encapsulated in PLGA microspheres conserved only about 5% of its initial biological activity, after the protein was released for 24 h in 154 mM of PBS, pH 7.4 [44].

Here, the specific *in vitro* antiviral activity of the burst-released IFN- α 2b closely matched that of the control, which were 130 ± 40% and 110 ± 40% respectively. This result indicates that the bioactivity of the IFN- α 2b that passively diffuses out during the first day remained unchanged after the encapsulation process (Figure 1). This is in agreement with the afo-

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 Alonso MJ, Gupta RK, Min C, Siber GR, Langer R. Biodegradable microspheres controlled-release tetanus toxoid delivery systems. Vaccine 1994;12:299-312. rementioned results using IFN- γ [35]. Nevertheless, to verify this observation we also assayed the *in vitro* released IFN- α 2b with a sandwich-type ELISA that uses specific IgG1 MAbs (free CBIFNA 2.3 and horseradish peroxidase-conjugated CBIFNA 2.4) against IFN- α 2b. This ELISA can accurately quantify biologically active IFN- α 2b discriminating it from nonnative IFN- α 2b and it also correlates with the antiviral bioactivity assay [21]. The capacity of the released IFN- α 2b recognized by this ELISA was completely conserved (data not shown), as expected.

IFN- α 2b extraction from the microspheres

The positive results seen with the passive diffusiongenerated IFN- α 2b preparations do not completely rule out the possibility of microsphere encapsulationassociated instability events (*e.g.*, degradation, inactivation of IFN- α 2b). Such instabilities need to be considered when developing any microsphere-based delivery system for bioactive IFNs [43, 44]. Therefore, we wanted to evaluate whether the recovery of IFN- α 2b would significantly increase above 20%, having thus access to a higher proportion of the IFN- α 2b encapsulated within the microspheres for further analysis. Characteristics of IFN- α 2b samples and controls prepared to carry out this evaluation, using the solvent extraction procedure detailed in Materials and Methods, are described in table 1.

Compared to passive diffusion, the recovery of IFN- α 2b from microspheres using the solvent extraction process increased from $20 \pm 5\%$ to about $51 \pm$ 4% (Table 1, sample A). The PLGA polymer did not interfere with the microBCA assay for IFN- α 2b, since a non-significant signal was detected when assaying the empty microsphere samples after extraction (sample B in table 1). Nevertheless, the PLGA polymer might interact with IFN- α 2b preventing a quantitative (higher than 90%) recovery of the protein. In favor of this idea are the following facts: (i) IFN- α 2b was not lost due to resuspension in the solvent mixture (dichloromethane and acetone (1:3 v/v) (sample C in table 1); $96 \pm 4\%$ of IFN- α 2b was recovered after the extraction of freeze-dried IFN- α 2b (sample C in table 1). This value was almost the same as that $(99 \pm 4\%)$ obtained for an equivalent sample of freeze-dried IFN- α 2b (D in table 1) that was directly dissolved (nonextracted) in PBS, pH 7.2. (ii) The recoveries for control samples such as empty microspheres mixed with free IFN- α 2b (E in table 1) and a freeze-dried mixture of empty microspheres and non-encapsulated IFN- α 2b (F in table 1) were $100 \pm 5\%$ and $80 \pm 6\%$, respectively. The recovery associated to sample F was lower than that corresponding to the other controls (C and E in table 1). This might suggest that the freeze-drying process induced IFN-a2b-PLGA interactions of unknown nature. In fact, other authors have found that hydrophobic contacts between the protein and the polymer may become more pronounced during or after the drying process [47]. Despite this, the recovery for sample F was higher than the value obtained for the microspheres containing IFN- α 2b (sample A in table 1).

Analyzing the solvent-extracted protein preparation may also contribute in inferring the characteristics of the IFN- α 2b within the microspheres, after the en-



Figure 7. RP-HPLC profile of passive diffusion-recovered IFN- α 2b. A) Interferon released from the microspheres during the first day. B) Control interferon incubated in the receiving fluid at 37 °C for 24 h.

capsulation process. According to SDS-PAGE, the IFN- α 2b extracted from the microspheres had 5.3% dimer; this value was similar to the percentage calculated for controls, which ranged between 4.4 and 6.4% (Figure 9). On the other hand, the RP-HPLC-based elution profile of the extracted IFN- α 2b showed a single major peak at retention time of 36.24 min, and this was very similar to the one corresponding to sample E (free IFN- α 2b mixed with empty microspheres followed by solvent extraction) in table 1 (Figure 10). The other controls under evaluation (C, D and F in table 1) had similar chromatographic profiles (data not shown). These results are comparable with those published by

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Figure 8. SDS-PAGE under non-reducing conditions of IFN- α 2b diffused out from the PLGA microspheres during 24 h at 37 °C (lane 1). The control was interferon incubated under the same conditions (lane 2).

Johnson *et al.* when they encapsulated human growth hormone in PLGA microspheres made by the cryogenic process [18].

Several w/o/w emulsification-associated factors are clearly known to act against the stability of PLGAencapsulated proteins [47]. Among them are: (*i*) the exposure of protein to organic solvent, (*ii*) the formation of aqueous/organic interfaces, (*iii*) the adsorption of protein on the PLGA matrix, and (*iv*) a low pH generated during the polymer degradation process. Therefore, one might expect a variety of instabilities (*e.g.*, denaturation, unfolding, aggregation) that produce



Figure 9. SDS-PAGE under non-reducing conditions of IFN- α 2b extracted from the PLGA microspheres (lane 1). The controls are: extraction of the IFN- α 2b mixed with empty microspheres (lane 2), extraction of the freeze-dried mixture of IFN- α 2b with empty microspheres (lane 3), freeze-dried IFN- α 2b dissolved in PBS, pH 7.2 (lane 4), extraction of freeze-dried IFN- α 2b (lane 5). The last lane (6) corresponds to a protein molecular mass marker. The values placed on the dimer bands correspond to the percentage of these fractions, related to the amount of the native IFN- α 2b bands.



Figure 10. RP-HPLC profile of solvent-extracted IFN-α2b. A) IFN-α2b extracted from the microspheres. B) Extraction preparation of free IFN-α2b mixed with empty microspheres. Chromatography was carried out with a 5 μm octyl C8 column.

adverse effects on the biological properties of the encapsulated IFN- $\alpha 2b$.

We found here that the solvent-extracted IFN- α 2b (sample A in table 1) was immunorecognized by the anti-IFN MAbs (CBIFNA 2.3 and CBIFNA 2.4) only

in $43 \pm 4\%$ (Figure 11A). None of the control samples showed this significant drop in the immuno-reactivities against these specific monoclonal antibodies. The freeze-dried IFN- α 2b either dissolved in PBS (sample D in table 1) or extracted (sample C in table 1) was highly immunorecognized (111 ± 1 and 92 ± 8%, respectively). The extraction preparation of IFN- α 2b mixed with empty microspheres (E in table 1) was also fully (100 ± 8%) immunorecognized. Even an extensively treated IFN- α 2b (mixed with empty microspheres and followed by freeze-drying) showed an immunorecognition as high as 83 ± 1% (Figure 11A).

Table 1. Samples and controls prepared for the characterization of the IFN- α 2b extracted from the microspheres.	
All samples were assayed for protein quantification and analyzed by SDS-PAGE, RP-HPLC, ELISA and antiviral	
activity test	

Sample	Composition		Treatment	Purpose	Recovery (%)
	IFN-α2b (µg)	MS* (mg)			
MS-IFN-α2b** (A)	160	20	Extraction	Sample under investigation	51 ± 4
Empty MS (B)	-	20	Extraction	To verify that the MS components would not give any additional (interfering) signal in the analysis.	ND***
IFN-α2b freeze- dried (C)	160	-	Extraction	To learn the solubility behavior of IFN- $lpha 2b$ in the solvent mixture	96 ± 4
IFN-α2b freeze- dried (D)	160	-	Dissolution in PBS pH 7.2	Reference in terms of the amount and initial characteristics of freeze-dried IFN- $\alpha 2b$ used in the experiments	99 ± 4
Empty MS mixed with IFN-α2b (E)	160	20	Extraction	To learn if there were interactions between the polymer and the IFN-α2b and if the extraction procedure induces changes in the protein	100 ± 4
Freeze-dried mixture of empty MS and IFN-α2b (F)	160	20	Extraction	To learn if there was any effect of the freeze-drying process on the potential interactions between the polymer and IFN- α 2b and if the extraction procedure induces changes in the protein	80 ± 4

*MS - Microspheres

**MS-IFN- α 2b - Microspheres containing IFN- α 2b

***ND - Non determined

A behavior similar to that of IFN- α 2b-associated immunorecognition was found for the *in vitro* antiviral activity of IFN- α 2b (see Figures 11 A and B for comparison).

These observations are consistent with previous studies in which protein (e.g., lysozyme) released as an initial burst was found to remain unchanged after the encapsulation process, while the protein population remaining within the microspheres was modified [31, 32, 47]. As stated above, the possible causes of protein modification are well ascribed to microsphere preparation steps, such as protein loading, microsphere formation, and drying ([47] and citations therein). These include: the formation of water/organic solvent interfaces, sonication-provoked cavitation stress, loss of hydrating water shell, freezing, pH shifts, among others ([47] and citations therein). Full preservation of the native protein structure still remains a major challenge in the development of PLGA-based microsphere delivery systems for active proteins [47]. A variety of approaches to overcome this problem (*i.e.*, to stabilize encapsulated IFN- α 2b and to improve its release profile) are available ([48] and citations therein), including (i) the chemical modification or physical blending of PLGA with hydrophobic monomers and polymers (e.g., polyethylene glycol, poly (ethyleneco-vinyl acetate), polyvinyl alcohol, composite PLGA, poly (acryloyl hydroxyethyl starch), or (ii) the use of hydrophilic particles or hydrogels (e.g., agarose, gelatin nanoparticles) for protein encapsulation prior to their conventional entrapment into the PLGA matrix. Other solutions have been excellently reviewed recently [47].

Given this, further research will continue to find optimized conditions to counteract the factors that compromise the integrity of IFN- α 2b during encapsulation and release.

Conclusions

IFN- α 2b (Center for Genetic Engineering and Biotecnology, Cuba) encapsulated by means of the double emulsion-solvent evaporation method into biodegradable PLGA microspheres showed reproducible characteristics such as morphology (spherical particles exhibiting a smooth surface with few pores), particle



Figure 11. Biocharacteristics of the solvent-extracted IFN- α 2b. The values refer to the percentage of the total interferon extracted that was immunorecognized by specific anti-IFN- α 2b monoclonal antibodies (A) or the percentage that conserved in vitro antiviral activity (B). These values were calculated with respect to the interferon mass (extracted or resuspended) that was estimated using the microBCA assay. For the antiviral activity calculation, the primary results (given in IU) were converted into interferon mass values using the specific activity for this IFN- α 2b (2 x 10⁸ IU/mg). Sample B corresponding to empty microspheres was not evaluated (see table 1) for details sample.

size (28.1 ± 0.4 µm), PLGA recovery (86 ± 1%), protein loading (*e.g.*, 0.60-0.67% for 10 mg/mL aqueous IFN- α 2b solution) and encapsulation efficiency (*e.g.*, 54-63% using 10 of mg IFN- α 2b/mL). The characteristics (particle size and IFN- α 2b loading) of the microspheres and of the process (encapsulation efficiency) strongly depended on the experimental conditions used (*e.g.*, stirring speed during the second emulsification step, protein concentration and volume of the internal aqueous phase). The microspheres prepared using 14 000 rpm and 100 µL of IFN- α 2b at 20 mg/mL had the best characteristics for further analyses.

From *in vitro* passive diffusion experiments for 28 days, the encapsulated IFN- α 2b did not show bioerosion-dependent profiles but exhibited a two-stage release pattern. The diffusion-controlled IFN- α 2b released was about 25 ± 5%. The burst period (20 ± 5%) occurred during the first 24 h of incubation. This incomplete release seems to be associated with a small degradation of the polymer, under our experimental conditions. The burst release-associated IFN- α 2b

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kept its initial physical, immunochemical and biological properties unchanged as determined using slab-PAGE, RP-HPLC, ELISA, and appropriate antiviral assays.

The solvent extraction of the encapsulated IFN- α 2b increased protein recovery from 20 ± 5 to 51 ± 4%. The slab-gel electrophoretic and RP-HPLC chromatographic profiles of the encapsulated IFN- α 2b remained unchanged compared to the controls. However, the immunorecognition and antiviral activity of the IFN- α 2b decreased to 43 ± 4% and 61%, respectively. Further research is necessary on encapsulation conditions to optimize the preservation of the biological properties of IFN- α 2b.

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