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Design, optimization, and *in vitro* characterization of idebenone-loaded PLGA microspheres for LHON treatment

Rubén Varela-Fernández [a,b], Carlos Bendicho-Lavilla [a,f,g], Manuel Martin-Pastor [c], Rocío Herrero Vanrell [d], María Isabel Lema-Gesto [b], Miguel González-Barcia [e], Francisco Javier Otero-Espinar [a,f,g,*]

[a] *Department of Pharmacology, Pharmacy and Pharmaceutical Technology, University of Santiago de Compostela (USC), Campus vida, Santiago de Compostela Zip Code: 15782, Spain*
[b] *Clinical Neurosciences Group, University Clinical Hospital, Health Research Institute of Santiago de Compostela (IDIS), Travesía da Choupana s/n Santiago de Compostela Zip Code: 15706, Spain*
[c] *Magnetic Resonance Unit, Infrastructure Supporting Network of Research and Technological Development, University of Santiago de Compostela (USC), Campus vida, Santiago de Compostela Zip Code: 15782, Spain*
[d] *Innoftal Research Group (UCM 920415), Department of Pharmaceutics and Food Technology, Faculty of Pharmacy, Complutense University, Madrid Zip Code: 28040, Spain*
[e] *Clinical Pharmacology Group, University Clinical Hospital, Health Research Institute of Santiago de Compostela (IDIS), Travesía da Choupana s/n Santiago de Compostela, Zip Code: 15706, Spain*
[f] *Institute of Materials iMATUS, University of Santiago de Compostela (USC), Campus vida, Santiago de Compostela, Zip Code: 15782, Spain*
[g] *Paraquasil Group, Health Research Institute of Santiago de Compostela (IDIS), Travesía da Choupana s/n Santiago de Compostela, Zip Code: 15706, Spain*

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ABSTRACT

Biodegradable poly(lactic-co-glycolic acid) microspheres (PLGA MSs) are attractive delivery systems for site-specific maintained release of therapeutic active substances into the intravitreal chamber. The design, development, and characterization of idebenone-loaded PLGA microspheres by means of an oil-in-water emulsion/solvent evaporation method enabled the obtention of appropriate production yield, encapsulation efficiency and loading values. MSs revealed spherical shape, with a size range of 10–25 μm and a smooth and non-porous surface. Fourier-transform infrared spectroscopy (FTIR) spectra demonstrated no chemical interactions between idebenone and polymers. Solid-state nuclear magnetic resonance (NMR), X-ray diffractometry, differential scanning calorimetry (DSC) and thermogravimetry (TGA) analyses indicated that microencapsulation led to drug amorphization. *In vitro* release profiles were fitted to a biexponential kinetic profile. Idebenone-loaded PLGA MSs showed no cytotoxic effects in an organotypic tissue model. Results suggest that PLGA MSs could be an alternative intraocular system for long-term idebenone administration, showing potential therapeutic advantages as a new therapeutic approach to the Leber's Hereditary Optic Neuropathy (LHON) treatment by intravitreal administration.

1. Introduction

Leber's Hereditary Optic Neuropathy (LHON) is a hereditary mitochondrial neurodegenerative disease, characterized by retinal ganglion cells (RGCs) degeneration, accompanied by an optic nerve functionality progressive loss (Behbehani, 2007). The disease's etiology is unknown, although all cases presented a maternal hereditary pattern, based on the presence of mitochondrial DNA point mutations. These mutations change the mitochondrial transport chain's complex I coding process, causing a decreased ATP synthesis, high oxidative stress levels and damaged glutamate transport, actions that lead to RGCs dysfunction and, consequently, to apoptotic cell death.

* Corresponding author at: University of Santiago de Compostela, Department of Pharmacology, Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, Campus vida s/n, 15782 Santiago de Compostela, Spain.
E-mail addresses: rubenvf1@gmail.com (R. Varela-Fernández), carlos.bendicho@gmail.com (C. Bendicho-Lavilla), manuel.martin@usc.es (M. Martin-Pastor), rociohv@farm.ucm.es (R. Herrero Vanrell), mariaisabel.lema@usc.es (M.I. Lema-Gesto), miguel.gonzalez.barcia@sergas.es (M. González-Barcia), francisco.otero@usc.es (F.J. Otero-Espinar).

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The interval between RGC dysfunction and cell irreversible damage may be considered as a chance where vision might be repaired by regularizing mitochondrial function. Currently, LHON treatment options remain limited. New generation CoQ10 analogs were developed with the purpose of increasing the pharmacophore bioavailability and maximizing its therapeutic potential (Newman and Biousse, 2004). Idebenone is a short-chain benzoquinone and an enzyme NADPH quinone oxidoreductase cofactor, endowed with a powerful antioxidant and inhibitory lipid peroxidation action, being able to protect cell membranes and mitochondria from oxidative damage. A few years ago (2015), idebenone was commercialized under the brand name of Raxone® (Santhera Pharmaceuticals, Pratteln, Switzerland), an orally administrated pharmaceutical form (film-coated tablets). It was administrated at high doses (900 mg/day for 24 weeks) for LHON treatment with low efficacy and great changeability because of the strain to reach active concentrations at the retinal level (Newman and Biousse, 2004). Nowadays, there is no available commercialized medicine for this disease.

In recent years, technological advancements have given rise to different novel drug delivery systems (DDS) into marketable distribution. Biodegradable polymer-based carriers may be currently considered as controlled and site-specific DDS for a predetermined time period, ranging from hours to months (Langer, 1998).

Currently there are few commercial formulations specifically intended for the treatment of retinal or posterior segment diseases (Varela-Fernández et al., 2020). Formulations such as Vitrasert®; Retisert®, Ozurdex®, Eylea®, Jetrea® or Lluvien® can be mentioned, among others. Most of them are commercialized as non-biodegradable or biodegradable intraocular implants or intravitreal solutions. Implants has the advantage of their prolonged drug release but microparticles can be intraocular delivered by a simple injection, and their composition and morphological properties can markedly influence their *in vivo* behavior, including drug release (Herrero-Vanrell and Molina-Martinez, 2007). Microencapsulation is known as one of the main drug-coating processes, leading to the obtention of free-flowing micrometric particles (MPs) with a 1–1000 μm range size, enabling drug stabilization and protection from the environment (Varela-Fernández et al., 2020). Among them, microspheres (MSs) were further introduced as new controlled DDS, being spherical particles with monolithic structure, where the drug is incorporated into the polymeric matrix. These systems show high selectivity for the target tissue and a controlled-and-sustained release ratio coupling, as well as high biocompatibility and bioavailability, minimizing possible side effects (Langer, 1998).

Oil-in-water (O/W) emulsion/solvent evaporation is the most commonly used method due to its ease and the chance of preparing MPs with particular and tunable features. This technique is mainly grounded on the obtention of a simple oil-in-water emulsion (O/W), where the active substance and the polymer are included into the oil phase. The emulsification is made by adding the organic phase into the external aqueous phase, which contains the surfactant to avoid caking or coalescence processes. The organic solvent is subsequently evaporated at room temperature, leading to the manufacture of drug-loaded MPs that can be collected by filtration (Kumari et al., n.d.).

Biodegradable polymers are of major awareness in microencapsulation procedures due to their easy removal from the body as non-toxic metabolites through innate hydrolysis reactions of ester bonds, by contact with biological or artificial fluids. A few biodegradable synthetic polymers, particularly linear polyesters such as poly (lactic-co-glycolic acid) (PLGA), approved by the Food and Drug Administration (FDA) for human use, are of prime interest in the design of carrier-based DDS. These polymers are usually soluble in organic solvents (Saez et al., n.d.), including dichloromethane, ethyl acetate, chloroform, acetone or tetrahydrofuran, among others.

PLGA is a thermoplastic aliphatic polyester (Jain, 2000) that shows many of the ideal properties of a microscale DDS. It provides a long-term delivery of the encapsulated drug and it degrades into biocompatible products that are further excreted by the normal physiological pathways (Garvin and Feschuk, 2005). PLGA also eases the MSs features customization (size, distribution size, surface, and charge, among others). Despite that, the hydrophobicity of the polymer straightly correlates with the drug ocular activity (Karataş et al., 2009).

PLGA microspheres can be an excellent candidate to prepare idebenone-loaded DDS for LHON treatment due to the drug physicochemical properties (Mw 338.4 g/mol, LogP 4.3, and poor aqueous solubility 0.00747 mg/mL), as well as the ease of obtaining biodegradable microspheres of adequate size for intravitreal administration.

The aim of this work was to investigate novel intravitreal approaches for an idebenone controlled and sustained release as a new alternative LHON treatment to diminish the need for systemic drug administration or minimize the frequency of required ocular injections. The present work describes the design and development of idebenone-loaded PLGA MSs by the oil-in-water emulsion/evaporation technique, as well as the physicochemical characterization. The appropriateness of idebenone-loaded PLGA MSs formulations to control and maintain the idebenone release was also performed by acquiring a reliable preclinical basis through *in vitro* and *ex vivo* assays.

2. Materials

Resomer® RG 502 (Mw: 10000 Da; lactide:glycolide = 50:50) (CAS number: 26161–42-2), Resomer® RG 502H (Mw: 17000 Da; lactide:glycolide = 50:50) (CAS number: 26780–50-7), Resomer® RG 503 (Mw: 24000 Da; lactide:glycolide = 50:50) (CAS number: 26161–42-4), and Resomer® RG 503H (Mw: 38000 Da; lactide:glycolide = 50:50) (CAS number: 31213–75-9) were purchased from Evonik (Essen, Germany). Idebenone (CAS number: M0021480) was provided by Acofarma® (Terrasa, Barcelona). Polyvinyl alcohol (PVA) (CAS Number: 9002–89-5) was acquired from Sigma-Aldrich (St Louis, USA). Dichloromethane (CAS number: 75–09-2) was purchased from Labkem Labware SL (Vilassar de Dalt, Barcelona). Franz diffusion cells were bought from Viadriafoc (Madrid, Spain). All other chemical reagents were of analytical grade.

3. Methods

3.1. Idebenone-loaded PLGA microspheres: Preparation procedure

Idebenone-loaded PLGA MSs were prepared by an oil-in-water (O/W) emulsion/solvent evaporation technique, based on a method developed in combination with the UCM 920415 research group (Esteban-Pérez et al., 2020; Bravo-Osuna et al., 2018; Fernández-Sánchez et al., 2017), with minor modifications. Briefly, both phases were firstly prepared; the oil phase was composed of dichloromethane (DCM) (5 mL), idebenone (40 mg) and the polymer (400 mg), while aqueous phase was composed of a PVA solution (1% w/v) (15 mL). The maturation medium was also prepared, being a 0.1% (w/v) PVA aqueous solution (50 mL).

The inner phase was prepared by dissolving both components (polymer and drug) into the organic solvent through a vortex stirring (Vortex® Genius 3, VWR International) (Germany) until complete dissolution. The aqueous phase was then slowly added to the organic one, and a mechanic homogenization process (Ultra-Turrax T25, IKA® Laboratories) (Staufen, Germany) was applied to create the O/W emulsion under 8000 rpm for 30 s (in an ice bath to avoid DCM evaporation). The resultant emulsion was later added to the maturation medium for 3 h, in an incubation bath at 40 °C under magnetic stirring (100 rpm) to ease the organic solvent evaporation. MSs were then collected by filtration through a 25 μm nylon sieve and a 10 μm nylon filter respectively, to select the desired size interval. The resulting MSs were thrice washed, filtered, and subsequently vacuum-dried for a 24-h period. In order to point out, the entire process was carried out under no aseptic conditions. Asepsis is a fundamental factor in intraocular/periocular controlled DDS, so the sterilization process was conducted as a

final step.

3.2. Microspheres physicochemical characterization

3.2.1. Mean particle size, particle size distribution and surface charge

The mean particle size and particle size distribution were measured by two different methods: imaging analysis as a previous control method, and dynamic light scattering (DLS), being the confirmation method for both parameters.

In the imaging analysis method, MSs samples were fixed in a slide for the shape, surface, and size optical observation. Image subsets were defined and analyzed by using an Olympus BX60/SC100 optical microscope and an Olympus CellSens Entry software, respectively. A specific rule set was established as a way to standardize the measurement process. All formulations were analyzed in triplicate.

The MSs average diameter, size distribution and ζ potential were also analyzed by dynamic light scattering (DLS) by means of two different particle sizers. The particlés average size and size distributions were determined by using a Malvern® Mastesizer micro laser diffraction particle sizer, with an internal sampler, a measured beam obscuration above 12.0% and a residual value below 0.3%. MSs ζ potential was obtained through a Malvern® Zetasizer ZS instrument, using folded capillary cells.

The resulting data were processed by using a combination of Malvern Mastersizer/Zetasizer and GraphPad® Prism software. Prior to measurement, samples were dispersed in Milli-Q® water and ultrasonicated for 10 s to promote the sample's homogenization. Results were analyzed by a one-way ANOVA analysis and a Student's t-like test, for the different formulations. A Tukey's multiple comparison test would be also employed to assess the presence of statistically significant changes among the prepared formulations.

The size distribution was also assessed by obtaining the *span* value, a dimensionless measure of the particle's size distribution. DLS particle sizer's measurement range and resolution were adjusted according to the type of sample, varying from 2 to 60%, based on the tube opening diameter, depending on the MSs size.

The *span* value may indicate MSs distribution size considering $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ results from different MSs formulations. The larger the *span* value, the wider the particle size interval in the powder; the lower the *span* value, the narrower the particle size range. A span value of 0 would indicate a monodisperse particle size distribution. Mathematically, the span value may be calculated as presented, through the following equation (equation (1)):

$$span = \frac{d_{0.9} - d_{0.1}}{d_{0.5}} \quad (1)$$

where 90% of the particles show a diameter inferior to $d_{0.9}$ value, 10% of the particles less than $d_{0.1}$ value and 50% of the particles below $d_{0.5}$ value. The diffraction particle sizer may obtain an absolute value related to the number of particles per unit of volume for different size intervals. The resulting data may support initial distribution size values obtained from the DLS analysis.

3.2.2. Morphological evaluation

The MSs external morphology was assessed by scanning electron microscopy (SEM), after the vacuum-drying process. MSs were included on an adhesive metal plate and sheltered by sputter-coating with iridium particles. Samples were further observed under different magnifications with an analytical scanning electron microscope.

3.2.3. Production yield (PY)

MPs were recovered and subsequently weighted at the end of the preparation process. The PY of each batch was obtained by dividing the MSs weight (after filtration and drying) by the total amount of the components used in the formulation process (Leonardi et al., 2009), as presented (equation (2)):

$$PY(\%) = \frac{MSs\ \ weight}{Total\ \ amount\ \ of\ \ components\ \ in\ \ the\ \ formulation} \hat{A}\cdot 100 \quad (2)$$

Likewise, a standardization value was established after taking 9 randomized samples from different batches for each formulation to guarantee the resulting data and as a way to confirm the preparation method's reproducibility. Results were processed by a two-way ANOVA analysis to simultaneously study two different variables. A Tukey's multiple comparisons test was also applied to assess the existence of statistically significant differences among the prepared formulations.

Table 1
Specifications of the factorial design for the MSs optimization procedure.

Drug:Polymer Ratio (w/w)	PVA (%) (w/v)
1:1	0.5
	1
	2
1:5	0.5
	1
	2
1:10	0.5
	1
	2

3.2.4. Encapsulation efficiency (EE) and loading capacity (LC)

EE and LC of MSs were measured by UV–Visible spectrophotometry at a 279.0 nm wavelength. EE (%) was calculated by both direct and indirect methods (determination of encapsulated drug and free drug, respectively), in order to verify that the low solubility of the drug did not interfere with its determination by either method. Data were further processed by the following mathematical equation (equation (3))):

$$EE(\%) = \frac{Total\ \ amount\ \ of\ \ drug - Encapsulated\ \ drug}{Total\ \ amount\ \ of\ \ drug} \hat{A}\cdot 100 \quad (3)$$

LC values were obtained by a centrifugation technique, trailed by the free-drug measurement by UV–Vis spectrophotometry, after polymer precipitation (de Mello and Ricci-Júnior, 2011; Guerreiro et al., 2012). Briefly, 20 mg of MSs samples were dissolved into 2.5 mL dichloromethane, followed by a mechanic stirring process (Vortex®, VWR International) (Germany) to promote the polymer dissolution. Later, 5 mL of methanol were appended, and samples were subsequently vortexed for 1 min to foster polymer precipitation. Samples were then centrifuged (5000 rpm, 10 min) and the supernatant was subsequently collected. Idebenone was finally determined by UV–Vis spectrophotometry (λ = 279 nm). This procedure was repeated using 9 randomized batches for each formulation. Loading capacity was then calculated with the following mathematical equation (equation (4)):

$$LC(\%) = \frac{Weight\ \ of\ \ released\ \ drug\ \ from\ \ the\ \ MSs}{MSs\ \ Weight} \hat{A}\cdot 100 \quad (4)$$

Results were processed by a two-way ANOVA analysis to simultaneously study two different variables. A Tukey's multiple comparisons test was also applied to assess the existence of statistically significant differences among the prepared formulations.

3.3. Optimization of microspheres preparation procedure

Based on the MSs elaboration procedure, different key parameters were further modified in order to achieve the optimum values for the preparation process. Certainly, a factorial design was previously established, where different drug:polymer ratios (1:1 w/w to 1:10 w/w) and aqueous phase concentrations (0.5–2%) (see Table 1) were tested and subsequently assessed in terms of PY, EE and LC of the resulting MSs. It must be considered that only one parameter was modified during the

Table 2
Physical characteristics of the different PLGA polymers employed in the present study.

Resomer	Feed Ratio	Mw (KDa)	Inherent viscosity (dL/g)	Form	Termination	Acid number (mg KOH/ g PLGA)
502	50:50	7–17	0.16–0.24	Amorphous	Ester	≤ 1
502H	50:50	7–17	0.16–0.24	Amorphous	Acid	> 6
503	50:50	24–38	0.32–0.44	Amorphous	Ester	≤ 1
503H	50:50	24–38	0.32–0.44	Amorphous	Acid	> 3

elaboration process, keeping the other key factors constant. All experiments were performed in triplicate.

3.3.1. Effect of the polymer characteristics

The idebenone-loaded MSs prepared with Resomer® RG 502 and 503, which are characterized by esterified carboxyl end groups, and Resomer® RG 502H and 503H, which have shown free carboxyl end groups, were compared to assess the effect of the carboxyl end groups of the different PLGA polymers on the resulting MSs size. According to Cegnar et al. (Cegnar et al., 2004) (Cegnar et al., 2004), an increase in the polymer's hydrophilic properties having free carboxyl end groups leads to a reduction of the solubility in methylene chloride and, therefore, a reduction of the MSs size.

MSs particle size may be additionally disturbed by the molecular weight (Mw) and the inherent viscosity of the polymer (see polymer details in Table 2). An increase in the Mw and the inherent viscosity may cause an upsurge in the viscosity of the internal phase, leading to a bigger MSs size, as a tougher shearing force for emulsion droplets' disturbance is needed (Freitas et al., 2005; Mittal et al., 2007).

3.3.2. Effect of the drug:polymer ratio and PVA concentration

Three different drug:polymer ratios (1:1, 1:5, and 1:10) and three different PVA concentrations (0.5% w/v, 1% w/v, and 2% w/v) for each drug:polymer ratio were tested. Regarding the drug:polymer ratio assessment, it must be considered that all formulations were prepared following the same procedure, using different drug:polymer ratios, as previously presented in Table 1. The drug:polymer ratio was modified by maintaining the amount of polymer, surfactant, and phases proportions constant in all formulations, and changing the amount of drug.

Additionally, PVA concentration in the dispersant phase is also a key parameter for the MSs particle size. In the present study, predefined concentration values of PVA (see details in Table 1) were assessed to determine its influence on the MSs physicochemical characterization during the elaboration process. The presence of PVA promotes the emulsion droplets steadiness by diminishing the interfacial tension, avoiding MSs aggregation or coalescence phenomena (Freitas et al., 2005; Mao et al., 2007).

3.4. In vitro release study

The idebenone release rate from MSs was determined by UV–Visible spectrophotometry, where vertical diffusion cells were employed as holding structures. The preparation and performance of the assay, as well as the sampling and analysis of the results, were detailed previously (Varela-Fernández et al., 2021). To point out, vertical diffusion cells contained two chambers, a donor and a receptor chamber, both separated by a dialysis membrane of defined pore size (14000 Da). Once mounted, 2 mL of simulated vitreous humor (0.1% w/v agar and 0.5% w/v hyaluronic acid aqueous solution) was added to the donor chamber followed by 1 mL of the idebenone-loaded MSs formulation (C_{MSs} = 25 mg/mL, C_{IDB} = 2.5 mg/mL), while the receptor chamber was filled with ocular buffer (PBS) (v = 7 mL). 1 mL of an idebenone suspension (C_{IDB} = 2.5 mg/mL) was added to the donor chamber instead of the microparticle formulation as a means of obtaining control data. The assay was run for a 50-day period, under controlled environmental conditions (100 rpm and 37 °C), and each formulation was tested in triplicate. Idebenone amount was measured using UV–Vis spectrophotometry at a 279.0 nm wavelength. Analytic validation was previously obtained by our group and exhibited linearity (R = 0.999) over a concentration interval of 0.19 – 250 μg/mL, 96.3% accuracy (1.85% RSD), of a 100.2% RSD intra-day precision and LOD and LOQ values of 0.3906 μg/mL and 0.7812 μg/mL, respectively. The resulting data proved that standard solutions remained stable for up to a week, protected from light exposure.

One-way ANOVA analysis was employed to determine time/group interactions among the MSs batches. A Tukey-Kramer Multiple Comparison Post-Test was run to establish the resemblance or variations concerning release profiles of selected formulations for cases where a significant interaction was found. Regression analysis of the *in vitro* release profiles was performed, and different mathematical models were fitted to formulation's release data by means of the GraphPad Prism® software.

The apparent thermodynamic solubility of idebenone in the release media was determined by adding excess idebenone to the media and then incubating for 24 h in an orbital shake (VWR mini shaker) under intense shaking. Samples were centrifuged 20 min at 11500 rpm (Eppendorf® Centrifuge 5804R) and idebenone concentration was measures by spectrophotometry (Agilent Cary 60). Each measurement was made in triplicate.

3.5. Stability studies

Stability studies involve a complex set of procedures that are of prime interest to evaluate efficacy, quality, and safety of drug-loaded pharmaceutical forms. Stability assessment is also considered a major focus of attention to carry out drug shelf-life prediction and determine the optimum storage conditions.

3.5.1. Stability to storage

The stability-to-storage study was established according to the ICH guidelines (Q 1 A (R2) Stability Testing of new Drug Substances and Products, 2006), with negligible changes. MSs were placed in screw-capped, amber-colored glass containers (5 mL capacity) and stored at three different temperature conditions (4 ± 2 °C, 25 ± 2 °C, and 37 ± 2 °C) for a three-month period. Stability was assessed through drug content uniformity. Drug content assay (n = 3 per batch) was carried out by weighing 20 mg of idebenone-loaded MSs, following the same procedure that has been applied for LC (%) determination. Drug content was finally estimated by UV–Vis spectrophotometry at a 279 nm wavelength.

Characterization of the structure of MS after 12 months stored in a cabinet in plastic containers at ambient temperature (17–25 °C) in absence of light was made using the techniques described in sections 3.7, 3.8, 3.10, and 3.11.

3.5.2. Stability to pH

The physical evaluation of the pH-dependent MSs stability is carried out on freshly prepared formulations. The methodology of this procedure is based on the suspension of 100 mg idebenone-loaded MSs in 5 mL of Milli-Q® water at different predetermined pH values (from 2 to 12) (Hanna® HI5522, Hanna Instruments®, Spain). Samples are then kept refrigerated (T = 4 °C ± 2 °C) for 24 h to be subsequently centrifuged at 5000 rpm for 10 min. Sink conditions have been ensured for the accomplishment of the test. Finally, the determination of the free idebenone concentration in the solution is carried out at a 279 nm

wavelength to verify the existence of MSs collapse or breakage phenomena with the consequent release of the drug into the medium. Each formulation is tested in triplicate for all set pH values.

3.5.3. Stability to ionic strength

The physical evaluation of the ionic strength-dependent MSs stability is carried out on freshly prepared formulations. The methodology of this procedure is based on the suspension of 100 mg idebenone-loaded MSs in 5 mL of NaCl aqueous solutions with different predetermined ionic strength values (from 0.2 M to 2 M). Samples are then kept refrigerated ($T = 4 \pm 2$ °C) for 24 h to be subsequently centrifuged at 5000 rpm for 10 min. Sink conditions have been ensured for the accomplishment of the test. Finally, the determination of the free idebenone concentration in the solution is carried out at a 279 nm wavelength to verify the existence of MSs collapse or breakage phenomena with the consequent release of the drug into the medium. Each formulation is tested in triplicate for all set ionic strength values.

3.6. Moisture content

The natural moisture content is an essential test for polymeric MSs, being defined as the ratio of the water weight compared to the weight of the solid materials in a certain mass of the formulation. Moisture content is assessed to ensure the quality of the formulations and to demonstrate that no significant stability changes occur during storage by hydrolysis of the components. Its determination was carried out by weight difference. For each formulation, 20 mg MSs were placed on an aluminum plate and dried at 105 °C until constant weight to completely guarantee water evaporation from the samples. The mass loss percentage was calculated as moisture content, where measurements were carried out in triplicate. Certainly, moisture content was mathematically estimated by using the following equation (equation (5)):

$$Moisture\ \ content(\%) = \frac{Initial\ \ MSs\ \ weight - Final\ \ MSs\ \ weight}{Initial\ \ MSs\ \ weight}\hat{A}\cdot 100 \quad (5)$$

3.7. Solid state nuclear magnetic resonance (ss-NMR)

Solid NMR spectra of the idebenone-loaded PLGA formulations were obtained at 298°K in a NEO-750 spectrometer (750 MHz proton frequency), equipped with a Varian T3 solid triple resonance probe 1H/X/Y with a zirconia rotor (3.2 mm outer diameter and a 22 μL effective sample capacity – approx. 30 mg of the powder sample -). The probe was adjusted to the Bruker spectrometer by an A2B conversion kit (Revolution NMR, LLC) and TopSpin® 4.0 software was used as the spectrometer control. All the NMR spectra were treated by using the MestreNova® software v14.0 (Mestrelab® Research Inc., Spain). Carbon chemical changes were referenced to the carbon methylene signal of solid adamantane at 28.92 ppm. Proton chemical shifts were externally referenced to one of the methylene protons of solid glycine (resonance: 4.1 ppm). The assignment of ^{13}C resonances of idebenone and PLGA was based on the predictions made with the ChemDraw® v14.0 software (Perkin-Elmer, Inc) for the covalent structures.

Direct polarization 1D ^{13}C-PARIS-xy spectrum of each sample was measured at MAS 20 kHz following the procedure previously described by Purusottam et al. (Purusottam et al., 2013), with slight changes. During the inter-scan relaxation delay right before the ^{13}C excitation pulse, a train of 25 μs and 12.5 kHz field strength PARIS-xy pulses were applied to introduce modulation sidebands at half the rotation rate causing the ^{13}C enhancement. The total duration of PARIS-xy irradiation was 3 s. The ^{13}C excitation pulse had a tilt angle of 90°and was applied with a 63.1 kHz B1 field strength. Heteronuclear decoupling throughout FID acquisition was carried out by the SPINAL-64 with a 79.4 kHz proton field strength. A 1 s inter-scan relaxation delay (d_1) and 512 scans were applied to finally obtained the spectrum.

Cross polarization 1D ^{13}C-CP-MAS spectrum of each sample ("hC.cp" sequence of the Bruker library) was acquired at MAS 20 kHz with 1000 scans and a 2.5 s inter-scan delay (d_1). Cross-polarization was operated for 2 ms with a 63.1 kHz constant carbon field strength, the power on the ^{1}H nucleus was linearly ramped from 70 to 100% with a 48.1 kHz peak field strength. Heteronuclear decoupling during the FID acquisition was performed by the Spinal-64 with a 79.4 kHz proton field strength.

3.8. Differential scanning calorimetry (DSC)

DSC is one of the most widespread thermal analysis procedures in powder characterization. Fusion, desolvation, recrystallization, decomposition or vitreous transition processes may be determined by this analysis technique (Clas et al., 1999; Verdonck et al., 1999). Possible interactions between idebenone and the polymers were studied by DSC, which was carried out by means of a TA Instruments® Q1000 DSC/TGA/IR analyzer (Madrid, Spain), calibrated with indium. 10 mg MSs were weighed and placed into sealed aluminum plates. DSC curves were obtained by a 10 °C/min scanning rate, conducted through a temperature interval that goes from 0 to 150 °C at a 10 °C/min heating rate, in a liquid nitrogen environment, using a 50 mL/min flow rate. Each formulation was tested in triplicate.

3.9. Thermogravimetry analysis (TGA)

Thermal gravimetry analysis is one of the main useful methods for the determination of the presence/absence of residual solvents in formulations prepared by the emulsion/solvent evaporation method. The schedule of the procedure is similar to that described for the DSC analysis. Briefly, approximately 10 mg MSs were loaded into aluminum plates and heated from 0 °C to 150 °C at a heating rate of 10 °C/min by using a TA Instruments® Q1000 DSC/TGA/IR analyzer (Madrid, Spain). Each formulation was tested in triplicate.

3.10. Fourier-transformed infrared spectroscopy (FTIR) analysis

Infrared absorption spectroscopy (IR) is the method used to determine the molecule structures with infrared-radiation absorption characteristics according to their molecular vibration. FTIR assay was carried out to determine MSs structural characterization. Certainly, MSs samples were scanned through the IR interval, ranging from 400 to 4000 cm^{-1}, using a 4 cm^{-1} resolution and dry air as a background blank to stabilize the FTIR system (GladiATR™, Varian Pike Technologies). The spectra of each sample were recorded at a 64 scans/min speed, obtaining 4084 different points, and resulting data were collected in transmittance values (%) and processed through *Resolutions Pro* software. Each formulation was tested in triplicate.

3.11. X-Ray diffractometry analysis

X-ray diffraction is one of the most commonly used methods in the study of solid materials, although it has also demonstrated a relevant application in the analysis of disordered states of matter. At present, this technique is moving towards the analysis of structured samples in submicron dimensions and towards the structural analysis of non-solid systems, by conducting a wide variety of studies including, not only qualitative and quantitative analysis of materials, but also microtextural, crystallinity, nonhomogeneous deformation analysis or phase change analysis, among others.

The X-ray diffractometry study was carried out following the ISO 9001: 2015 standard normative in terms of reception, sample management and analysis using monocrystalline, crystalline powder and X-ray fluorescence techniques. All samples were kept at room temperature, in vials, waiting to be measured. The obtaining and handling of the materials to be analyzed was carried out by using latex gloves, with an agate mortar for spraying / homogenizing the physical samples.

MSs crystalline structure was analyzed by wide-angle X (WAX) ray powder diffraction in an X-ray diffractometer (Philips X'Pert, USA), operated with a PW170 control unit, a PW1820/00 vertical goniometer and an Enraf Nonius FR590 generator, locating and assigning the most significant mathematical values of the peaks of the diffractograms present. Samples were scanned from a Cu- Kα source, monochromated with a graphite monochromator (λ = 1.5406 Å), at 40 kV and 30 mA, using 2θ from 2° to 50° at a scan rate of 0.04°·min^{-1}. Samples were rotated during the analysis to obtain the most optimal peak profiles for the diffractograms, as well as to minimize the effect of the preferred orientation. It also must be considered that samples were deposited in oriented glass bases (Si-511 plate) to avoid background noise caused by a vitreous support. The mathematical analysis of the diffractograms was carried out by using the HighScore Plus® 3.0d software.

3.12. Mercury intrusion porosimetry (MIP) assay

The porosity, also known as void fraction, of polymeric MSs is a critical factor influencing the release kinetics of encapsulated active substances (Mao et al., 2007; Klose et al., 2006; Vysloužil et al., 2014). Several approaches in the porosimetry determination of particulate DDS are grounded on previously established procedures used in other scientific fields (Volfkovich et al., 2005). MIP is one of the most common and informative methods based on the pore size and distribution measurements. Furthermore, MIP presents certified reference material and standard measurement protocols (Espinal, 2013).

Porosity measurements were made by mercury intrusion porosimetry, using a Micromeritics Autopore IV mercury porosimeter (Norcross, GA, USA), equipped with a 3-mL capacity penetrometer and a 0.004 to 172.4 MPa analysis pressure interval, leading to the acquisition of "Pore volume vs Pressure" data (Andhariya et al., 2017). The procedure's schedule was based on weighing 200 mg idebenone-loaded MSs and were added to a glass penetrometer, appropriate for powder samples. Then, the penetrometer was closed, and preparations were subsequently included into the porosimeter, using a 0.004 MPa filling pressure for testing. Total intrusion volume, total pore area and porosity were recorded. Porosity was calculated by two different methods: 1) as the quotient of pore volume to total MPs volume and 2) as the quotient of bulk density and apparent density as presented (equation (6)):

$$Porosity(\%) = 1 - \frac{bulk\ \ density}{apparent\ \ density} \hat{A}\cdot 100 \quad (6)$$

Mercury intrusion into each formulation was also analyzed by means of the Washburn equation (equation (7)) to estimate the pore diameter distribution, and resulting data were recorded on the PoreXpert™ software.

$$Washburn\ \ equation: D = -\frac{4\gamma}{P} \hat{A}\cdot \cos\theta \quad (7)$$

where D represents the pore diameter, γ refers to the surface tension of Hg at 20 °C, P embodies the applied pressure, and θ denotes the contact angle between Hg and the powder, which is assumed to be 130° (Washburn, 1921).

Pore volume values were described as the amount of volume per gram (cm^3/g) and obtained by using polymer density and sample weight of the porosimetry sample. Total MSs volume was measured as the sum of the pore volume and the polymer volume (Reinhold and Schwendeman, 2013).

3.13. Syringeability and injectability studies

Syringeability and injectability are two important features in the preclinical evaluation of MSs formulations for intraocular dosage forms. The former denotes the ease of passage of a formulation to easily traverse a needle from an appropriate container prior to a syringe system, while the latter alludes to the procedure of the MSs suspension throughout the injection process (Cilurzo et al., 2011). Suitably syringeability and injectability properties guarantee the right dose of MSs to be intraocularly administered.

Table 3
Injectability and syringeability conditions for MSs formulations test.

Condition	Parameter	Injectability conditions	Syringeability conditions
System	Assay mode	Simple	Simple
	Assay type	Compression	Traction
	Force polarity	Compression	Compression
	Force sense	Downwards	Upwards
Sensor	Force scale	1000 N	1000 N
	Displacement	500 mm	500 mm
Assay	Action	Force	Force
	Control	Displacement	Displacement
	Sampling	10 seg/sample	10 seg/sample
	Breakage sensitivity	10%	10%
	Sensor	Displacement	Displacement
Sample	Material	–	–
	Shape	Cylinder	Cylinder
	Dimensions	Diameter and height	Diameter and height
Data	Maximum force	–	–

Special care must be taken when preparing homogenous particle dispersion of MSs formulations intended to be injected as a conventional suspension in clinical practice. Buffer solutions, such as pH 7.4 Balanced Saline Solutions (BSS) or Phosphate Balanced Solutions (PBS), are mainly used as delivery vehicles for their biocompatibility, transparency, ability to rapidly dilute in the intraocular fluids and easy removal from the eye. Syringeability and injectability studies of MSs suspensions enable to establish the optimal characteristics of the intraocular needles (size and length), by calculating the needed force to inject a predetermined volume of the formulation. A 12 N force over 10 s was set as the maximum ejection force, being appropriate for proper intraocular injection.

Sample's preparation procedure was based on the elaboration of 10 mg MSs/mL suspensions by sonication (10% intensity for 10 s; Sonopuls® LS 40/HG 40 2000.2 series) (Berlin, Germany), where MSs were incorporated into a 0.1% (w/v) PVA aqueous solution. Once prepared, MSs suspensions were included in 5 mL syringes, equipped with different types of needles (21G, 27G and 30G), in order to compare them for an appropriate administration (Xie et al., 2014). Ejection and aspiration forces were respectively obtained by using a universal testing machine (Shimadzu® AGS-X Series, Kyoto, Japan), managed through a specific software (Trapezium®). Established injectability and syringeability conditions were as presented in Table 3. All the samples were tested in triplicate for all the different syringe/needle systems.

3.14. Ocular irritancy and toxicity analysis of microspheres: Hen's egg test on the chorioallantoic membrane (HET-CAM)

The general test for substance irritation and corrosion is based on the rabbit eye test settled by Draize et al. (Wallig et al., 2018) and has turn into the universal and traditional assay for acute eye irritation and corrosion. Nevertheless, its cruelty gave way to several authorized alternative tests, showing potential as screens for ocular irritancy, such as the HET-CAM test (Luepke, 1985).

In the HET-CAM test, three different phenomena are assessed (hemorrhage, lysis, and coagulation). The protocol was improved from the method formerly explained by Spielmann and Liebsch (Spielmann et al., 1993), with negligible changes. The preparation and performance of the assay, as well as the image sampling were previously detailed (Varela-Fernández et al., 2021).

Briefly, fertilized Broiler eggs (obtained from the regional hatchery technology center, Coren Ourense) were incubated for nine days at 37 ±

Table 4
Physicochemical characterization of idebenone-loaded PLGA MSs. Production yield (PY), encapsulation efficiency (EE) and loading capacity (LC) are presented for all the tested conditions of the optimization procedure.

Formulation	Drug: Polymer Ratio	PVA (%)	PY (%)	EE (%)	LC (%)
502 MSs	1:1	0.5	54.56 ± 5.85%	98.21% ± 0.17%	60.95% ± 8.65%
		1	62.10 ± 5.50%	98.22% ± 0.10%	67.50% ± 5.85%
		2	78.10 ± 10.91%	97.65% ± 0.32%	71.01% ± 5.03%
	1:5	0.5	88.50 ± 0.88%	93.15% ± 0.45%	26.42% ± 3.51%
		1	85.62 ± 7.12%	93.55% ± 0.81%	35.48% ± 3.41%
		2	80.88 ± 4.93%	90.90% ± 0.86%	16.22% ± 2.58%
	1:10	0.5	87.89 ± 1.13%	85.64% ± 0.65%	21.62% ± 3.20%
		1	87.37% ± 2.30%	87.59% ± 0.78%	22.05% ± 2.52%
		2	86.96% ± 0.65%	87.00% ± 0.58%	18.88% ± 4.03%
502H MSs	1:1	0.5	85.68% ± 1.95%	97.79% ± 0.33%	60.95% ± 8.89%
		1	88.73% ± 0.62%	97.58% ± 0.46%	67.47% ± 3.56%
		2	85.28% ± 4.05%	97.50% ± 0.35%	71.42% ± 6.22%
	1:5	0.5	76.14% ± 0.54%	91.56% ± 0.82%	26.41% ± 2.83%
		1	83.94% ± 2.14%	92.18% ± 1.12%	35.31% ± 2.24%
		2	84.53% ± 2.85%	93.59% ± 0.36%	16.28% ± 3.62%
	1:10	0.5	83.61% ± 2.94%	86.85% ± 0.36%	21.12% ± 2.55%
		1	86.32% ± 0.86%	88.65% ± 1.32%	22.09% ± 1.46%
		2	80.60% ± 2.37%	84.69% ± 1.80%	22.21% ± 1.33%
503 MSs	1:1	0.5	80.38% ± 4.31%	97.25% ± 0.20%	68.63% ± 5.38%
		1	77.47% ± 3.55%	97.99% ± 0.12%	72.32% ± 6.40%
		2	81.59% ± 1.08%	97.34% ± 0.14%	71.39% ± 4.72%
	1:5	0.5	87.91% ± 1.56%	91.93% ± 0.28%	28.60% ± 1.73%
		1	87.57% ± 1.13%	93.79% ± 0.20%	38.36% ± 6.88%
		2	84.74% ± 4.19%	88.21% ± 0.24%	23.48% ± 2.62%
	1:10	0.5	80.87% ± 1.80%	85.79% ± 2.04%	15.64% ± 3.01%
		1	83.64% ± 5.47%	88.40% ± 2.33%	17.01% ± 2.93%
		2	87.16 ± 1.28%	88.85% ± 0.55%	14.01% ± 1.51%
503H MSs	1:1	0.5	84.68% ± 1.64%	97.82% ± 0.89%	66.56% ± 5.93%
		1	87.98% ± 3.02%	98.02% ± 0.36%	69.44% ± 4.33%
		2	90.77% ± 3.05%	97.56% ± 0.10%	92.63% ± 7.56%
	1:5	0.5	79.76% ± 10.31%	92.70% ± 0.75%	28.79% ± 4.94%
		1	83.54% ± 3.35%	93.65% ± 0.75%	26.49% ± 1.74%
		2	84.70% ± 5.07%	93.04% ± 1.05%	18.17% ± 1.60%
	1:10	0.5	87.53% ± 1.66%	88.31% ± 0.41%	18.48% ± 2.43%
		1	85.68% ± 3.29%	89.20% ± 0.61%	13.94% ± 1.14%
		2	90.10% ± 0.59%	87.31% ± 0.65%	14.50% ± 1.72%

0.5 °C and 65%±5% RH. A window on the head of the eggs was open using a Dremel 400 multi-tool and the membrane that protects the chorioallantoic membrane (CAM) was removed. A volume of 300 μL of MSs formulations (the same as used in syringeability and injectability studies), positive (1 M NaOH aqueous solution) or negative control (0.9% NaCl) were directly placed onto the CAM. The evaluation of the key parameters over a 5 min period was carried out using an Olympus SZ61TR Stereomicroscope and an Olympus CellSens Entry® software. Imaging analysis and processing were also carried out as detailed in previous works (Varela-Fernández et al., 2021). Each formulation was tested in triplicate.

3.15. Statistical analysis

All tests were performed, at least, in triplicate and obtained data were included as average values and their standard deviations. Likewise, if needed, statistical significance was calculated and included in the result's paragraph. Additional data analysis procedures may be applied for specific parameters. Different specific software was used to analyze all the collected data.

4. Results and discussion

4.1. Microsphere's preparation

The emulsion/solvent evaporation technique is a commonly employed methodology for microencapsulation to prolong and control the drug release from the DDS. In the present work, an O/W emulsion/solvent evaporation methodology was used to efficaciously entrap a hydrophobic drug (idebenone) into polymeric MSs, according to the component's data previously mentioned (see details in Table 1). First, idebenone was dissolved into a polymer organic solution and subsequently emulsified with a PVA aqueous solution. Polyvinyl alcohol (PVA) acts as an emulsifying agent (20,21) and presents unique characteristics, making it ideal for controlled drug delivery. It provides control of the MPs mechanical properties, degradation rate changes, and immune response with negligible toxicity (22). After organic solvent evaporation, polymer precipitation occurred and MPs solidified, entrapping the drug in its matrix. After vacuum-drying, all idebenone-loaded PLGA MSs showed a free-flowing powder aspect and orange color. In order to point out, idebenone-loaded PLGA MSs were named as 502 MSs, 502H MSs, 503 MSs and 503H MSs, depending on the PLGA physical characteristics (see details in Table 2).

4.2. Microsphere's optimization procedure

The optimization procedure for the MSs preparation was carried out to obtain the optimal parameters for the final formulations, according to the factorial design previously described (see details in section 3.3.). Table 4 includes the data for the PY, EE and LC values for all the formulations tested during the optimization procedure. All formulations showed suitable PY, EE and LC values. These data are supported by the idebenone low aqueous solubility, which provides high drug entrapment into polymeric MSs, as well as the drug:polymer proportion employed in the elaboration procedure.

The particle size and size distribution were similar for all the formulations due to the time fitting during the mechanic homogenization process to obtain the highest production yield for the different polymers.

Table 5
Final idebenone-loaded MSs formulations.

Formula	Polymer	Drug:Polymer Ratio	PVA (w/v)
F1	Resomer® RG 502	1:10	1
F2	Resomer® RG 502H	1:10	1
F3	Resomer® RG 503	1:10	1
F4	Resomer® RG 503H	1:10	1

Those parameters also seemed to be influenced by the Mw and the inherent viscosity of the polymer. Thus, smaller particles would be obtained with Resomer® 502 and 502H compared with Resomer® 503 and 503H for the same mechanical homogenization procedure, possibly due to the slightly physicochemical differences of the polymer properties. All the MSs batches were assessed in terms of PY and found to be in the range of 50–90% (see Table 4). Despite all, these production yield values might be underestimated due to the manual manufacturing procedure and might be related to the handling loss throughout the processing steps, including polymer stickiness to the glass container, mechanic homogenization time or MSs loss during the washing step, among others. Nevertheless, the resulting data were analyzed by one-way ANOVA, Brown-Forsythe's, Bartlett's and Tukey's tests and no-statistically significance was observed ($p < 0.05$) among all formulations.

The resulting data may also indicate that particle size may be influenced by the Mw and the inherent viscosity of the polymer. Formulations prepared with Resomer® RG 502 or 502H (lower Mw and poorer inherent viscosity) were compared with formulations prepared with Resomer® 503 or 503H, with higher molecular weight and inherent viscosity, and a particle size increase would be observed during the preparation procedure. Nevertheless, the procedure schedule's times were adjusted for the different polymers to maximize the PY of the idebenone-loaded MSs. Thus, the resulting data were analyzed by one-way ANOVA, Brown-Forsythe's, Bartlett's and Tukey's tests and no-statistically significance was observed ($p < 0.05$) among all the formulations.

Regarding idebenone-loaded PLGA MSs, two key parameters were also studied: (I) the PVA concentration in the external medium and (II) the drug:polymer ratio. An increase in the PVA concentration (from 0.5% to 2% (w/v)) did not produce a noteworthy modification in EE values ($p > 0.05$), but in the LC values ($p < 0.05$). It is expected that higher PVA concentrations in the dispersant medium enable a steadier emulsion's formation, preventing drug migration to the external phase in addition to increase the LC values (Mao et al., 2007; Yang et al., 2001). Nevertheless, when the ratio of polymer increases (1:5, 1:10), the PVA concentration barely affects LC or even decrease.

The effect of ester (more hydrophobic) and carboxyl (more hydrophilic) end groups of the PLGA on the drug LC was also assessed. The dominant force between the drug and the polymer may be usually a hydrophobic interaction, since hydrophobic polymers with ester end groups improve the obtention of EE and LC values for hydrophobic drugs. Nevertheless, in this specific case, no significant differences were observed between the four different PLGA polymers used.

Based on these results, just one formulation prepared with each of the different polymers was carefully chosen. Certainly, the final formulation specifications are presented in Table 5. The rest of the characterization assays would be subsequently performed in these formulations.

4.3. Microspheres physicochemical characterization

4.3.1. Mean particle size, particle size distribution and surface charge

Granulometric assays were performed for determining particle size, size distribution and production yield of the idebenone-loaded PLGA MSs. A predefined size interval makes MSs suitable for its intravitreal administration through a 30G needle, these being supported by previous studies (Herrero-Vanrell et al., 2014; Martínez-Sancho et al., 2004). Furthermore, different sieves and filters (from 10 to 25 μm) were used during the filtration process to preestablish the desired size interval.

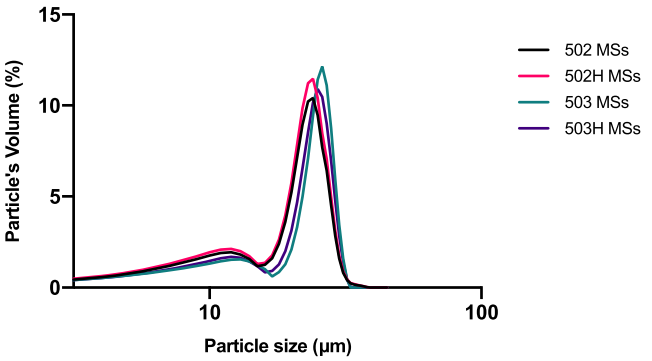

Fig. 1. Size distribution of selected idebenone-loaded PLGA MSs.

Table 6
Distribution size characterization by span value of the PLGA MSs.

Formulation	$d_{0.1}$	$d_{0.5}$	$d_{0.9}$	Span
502 MSs	1.58 ± 0.05	9.42 ± 0.56	19.41 ± 4.49	1.87 ± 0.34
502H MSs	1.72 ± 0.02	10.40 ± 0.60	19.47 ± 1.34	1.71 ± 0.07
503 MSs	1.92 ± 0.01	13.06 ± 0.05	23.20 ± 0.09	1.63 ± 0.01
503H MSs	1.80 ± 0.03	11.67 ± 0.33	22.18 ± 0.87	1.75 ± 0.02

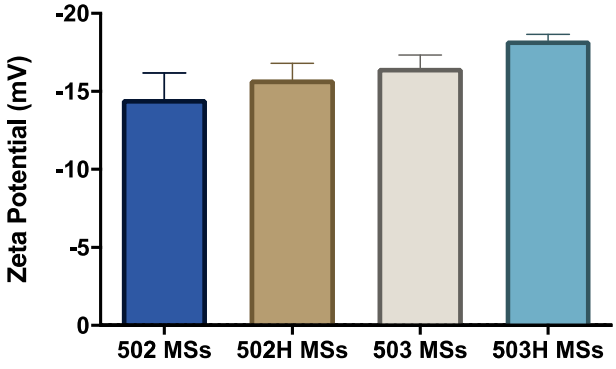

Fig. 2. Surface charge values of selected idebenone-loaded PLGA MSs.

Distribution size measurements may confirm that the preestablished filtration process is adequate for the obtention of an appropriate MSs size interval, as presented in Fig. 1. Likewise, size distribution was assessed through the span value estimation, as shown in Table 6. All the resulting span values also confirm a closely-monodisperse size distribution.

The particle size and size distribution were similar for all the formulations due to the time fitting during the mechanic homogenization process to obtain the highest production yield possible. Those parameters also seemed to be influenced by the Mw and the inherent viscosity of the polymer. Nevertheless, the resulting data were evaluated by a one-way ANOVA analysis and no statistically significance was observed ($p > 0.05$).

The MSs superficial charge was obtained by ζ potential determination. All formulations show an appropriate negative surface charge, as presented in Fig. 2. As per the Derjaguin, Landau, Verwey, and Overbeek (DLVO) electrostatic theory, this negatively charged net suggests that the formulations are stable over time because of Brownian motion and negative repulsive forces, desirable to prevent particle aggregation (Soni et al., 2014). Besides, this negativity might be attributed to PVA adsorption onto the MSs surface, functional group changes on the

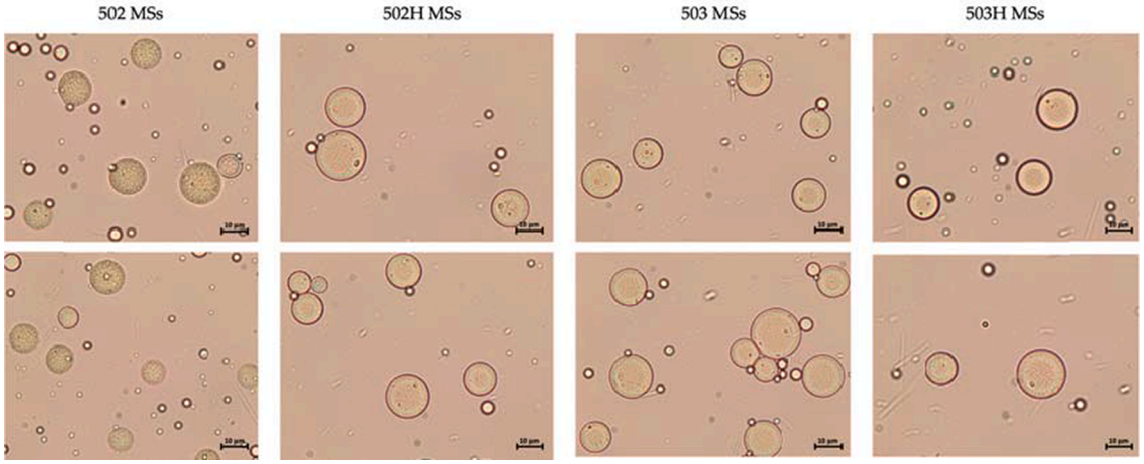

Fig. 3. Optical microscopy images of idebenone-loaded PLGA-based microspheres.

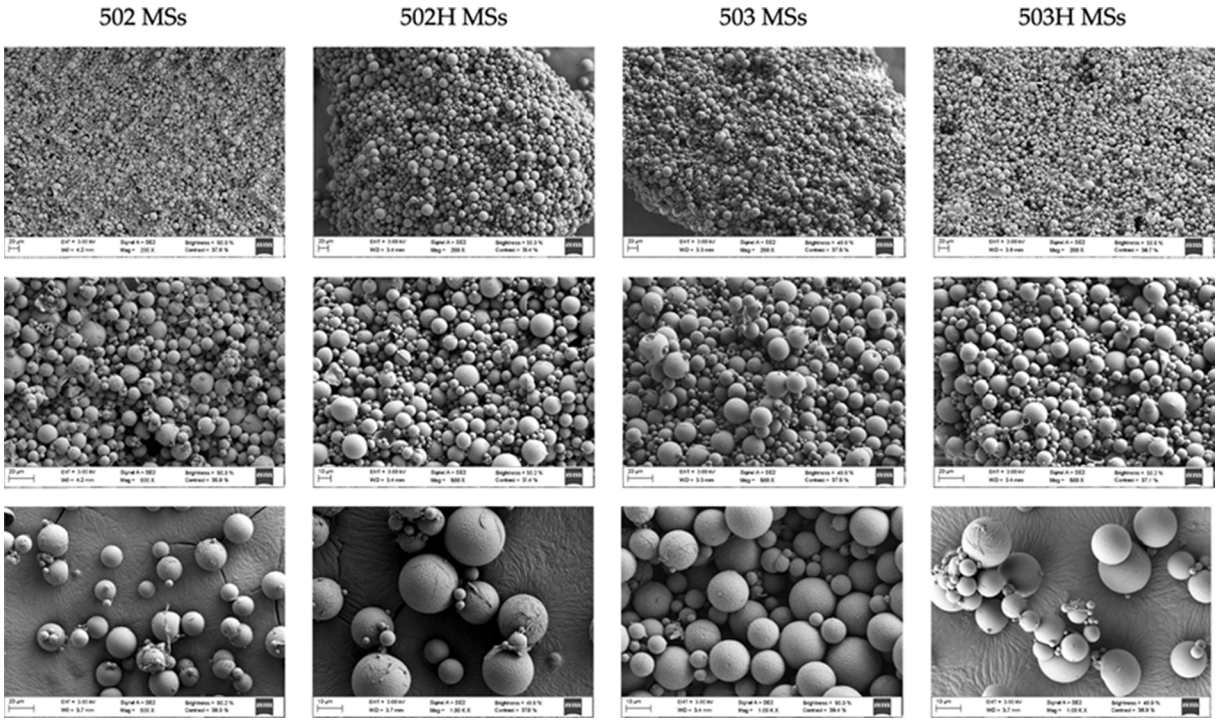

Fig. 4. SEM images of idebenone-loaded PLGA-based microspheres.

particle surface, or ionization of the PLGA carboxylic groups (Budhian et al., 2007).

One-way ANOVA analysis was submitted, and no statistically significant differences ($p > 0.05$) were observed among the different formulations (F value = 8.057, P-value = 0.0579, Geisser-Greenhouse's epsilon = 0.3677). A Tukey's multiple comparison test was subsequently carried out, and no differences were spotted among formulations, except for 502H and 503H formulations, possibly due to test experimental conditions.

4.3.2. Morphological evaluation

The morphological evaluation through optical microscopy was performed to relate the size and shape of the MSs. The microscopy images show spherical particles with a presumed internal granulation, observing uniformity in terms of morphology among the different types of polymers (see Fig. 3).

SEM evaluation was also performed to analyze MSs shape and surface, considering that MSs morphological characterization may also correlate particle size and surface characteristics with the rest of pharmacokinetic parameters. SEM observation demonstrated reliable results with previous optical observation, where MSs size distribution was narrow and adequate for intravitreal administration due to the selected size interval (see Fig. 4). The resulting idebenone-loaded PLGA MSs were without pores, as can be seen in SEM images. Images reveal the methylene chloride complete removal during the elaboration process.

The surface analysis demonstrated that blank and idebenone-loaded MSs were free flowing, with a diameter of 18.07 ± 4.13 μm, and revealed a spherical shape and relatively smooth surface, with micropores at high magnification, probably due to the slow release of dichloromethane. Furthermore, no detrimental effects were observed due to the drug loading.

Table 7
Encapsulation efficiency (EE) and loading capacity (LC) values of selected idebenone-loaded PLGA MSs.

Formulation	PY (%)	EE (%)	LC (%)
502 MSs	87.37 ± 2.30	87.59 ± 0.78	22.05 ± 2.52
502H MSs	86.32 ± 0.86	88.65 ± 1.32	22.09 ± 1.46
503 MSs	83.64 ± 5.47	88.40 ± 2.33	17.01 ± 2.93
503H MSs	85.68 ± 3.29	89.20 ± 0.61	13.94 ± 1.14

4.3.3. Production yield (PY), encapsulation efficiency (EE) and loading capacity (LC)

Table 7 shows the results for PY, EE and LC of idebenone-loaded PLGA MSs. All MSs batches showed suitable PY, EE and LC values for the applied preparation procedure.

One of the main benefits of the O/W emulsion/solvent evaporation procedure was the chance of preparing MSs with high PY values (over 80%) for all the selected formulations (see Table 7). Furthermore, a decrease in the polymer's inherent viscosity may cause a decrease in the amount of adhered-to-container polymer and, thus, increases the production yield. Final formulations were compared by a two-way ANOVA analysis, observing no statistically significant differences among the different formulations ($p > 0.05$).

The EE determination proved that the idebenone-loaded preparation process was reproducible and effective, with values above 85% in all formulations (see Table 7). The EE results were consistent for both the direct and indirect methods, and there were no significant differences between the two types of measurement. LC values ranged from 10 to 25% and, considering that a 1:10 drug:polymer ratio was employed, the resulting values are in line with expectations. These data are supported by the idebenone low aqueous solubility, which provides high drug entrapment into polymeric MPs, as well as the drug:polymer proportion used in the elaboration process.

A two-way ANOVA analysis was further submitted to the data to assess the presence or absence of differences among all formulations. The results show no statistically significant differences ($p > 0.05$) in terms of PY, EE and LC.

4.4. In vitro release study

Different mechanisms may be involved in the encapsulated drug release from PLGA MPs, where diffusion and biopolymer erosion are the main ones (Siegel et al., 2006; Kamaly et al., 2016). The diffusion rate is dependent upon drug diffusivity and partition coefficients (Swarbrick et al., 2001), although drug physicochemical properties, such as molecular size, hydrophilicity, and surface charge must be also taken into account (Swarbrick et al., 2001).

Likewise, hydrophobic drugs may hamper water diffusion into MPs as well as reducing the polymer degradation rate (Klose et al., 2006; Siegel et al., 2006). Hence, the effects of the encapsulated drug may be considered into the microparticulate systems' design due to its influence on the release mechanisms underlying biopolymer degradation (Siegel et al., 2006).

The two leading mechanisms involving drug delivery from PLGA MSs are mainly diffusion and polymer erosion (Kamaly et al., 2016). Generally, drug release from PLGA MSs arises into different stages. Firstly, a quick reduction in the polymer's Mw but a slight mass loss is observed while, in the subsequent stage, the contrary phenomenon happens, supporting the fact that PLGA degradation from MSs comprises mixed mechanisms, where drug delivery is primarily buttressed by diffusion processes rather than polymer degradation (Engineer et al., n. d.). Nevertheless, none of the different MSs formulations prepared in the present work have shown the initial burst release phase, leading to a more slow, stable, and constant release from the beginning of the *in vitro* release study.

PLGA polymers are typical bulk-eroding biopolymers where water readily infiltrates into their matrix, leading to pore formation into the MSs structure, where degradation processes take place (Varde and Pack, 2004). Upcoming research relies on achieving a better enlightening of this knowledge gap regarding the design of microparticulate systems (Engineer et al., n.d.), which may potentially be included into formulations encompassing heterogeneous release-profile biopolymers (Feng et al., 2015).

Drug release profiles from polymeric MPs were described to be harshly conditioned by PLGA properties (size, molecular weight, lactide: glycolide proportion or surface thermodynamics, among others), and by the mechanisms involved in the release process (Luan and Bodmeier, 2006; Sahana et al., 2008). In the present study, idebenone release from PLGA MSs seems to follow a monophasic pattern, characterized by a prolonged, uniform, and controlled release phase along the studied interval. Such idebenone release profile might be associated with polymer degradation processes, from drug diffusion through the matrix or even both simultaneously (Mittal et al., 2007; Holgado et al., 2009). The *in vitro* release profiles of the idebenone-loaded MSs formulations are shown in Fig. 5.

Very low dissolution kinetics of the IDB suspension was observed. The drug apparent thermodynamic solubility in PBS and simulated vitreous humor was 1.303 ± 0.002 and 1.304 ± 0.005 μg/mL respectively. The values indicate that no sink conditions were followed during the experiment and so the low dissolution rate observed in IDB suspension is mainly due to the saturation of the release media caused by the low drug solubility. In these no-sink conditions, a modified release of IDB from the microparticles is observed, reaching about 25% of drug released over 50 days. Additionally to the low drug aqueous solubility, it may be a concurrence of factors that can have influenced the MSs low drug release rate. Microparticles were deposited in the donor compartment, filled with vitreous humor, a medium of high viscosity that mimics the physicochemical characteristics of the physiological vitreous humor. Also, the donor chamber was not subject to agitation (only the receptor chamber was stirred to facilitate the renewal of the medium) to mimic as closely as possible the physiological state in the eye.

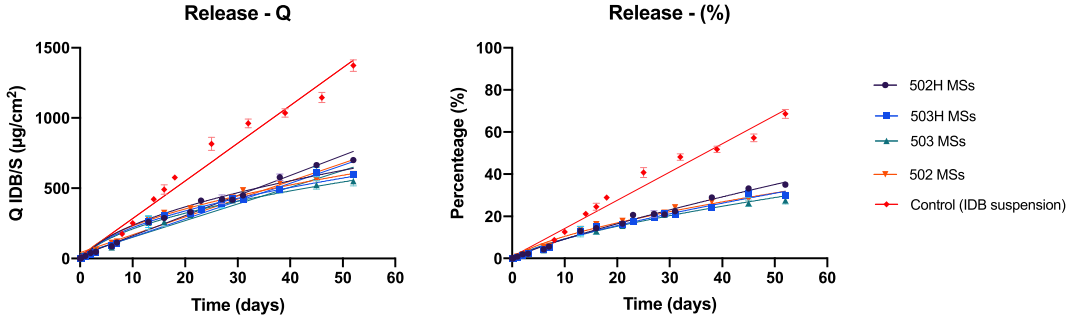

Fig. 5. *In vitro* release study of idebenone-loaded PLGA MSs. The profile of the idebenone-loaded PLGA MSs was compared with an idebenone aqueous suspension containing the same amount of drug.

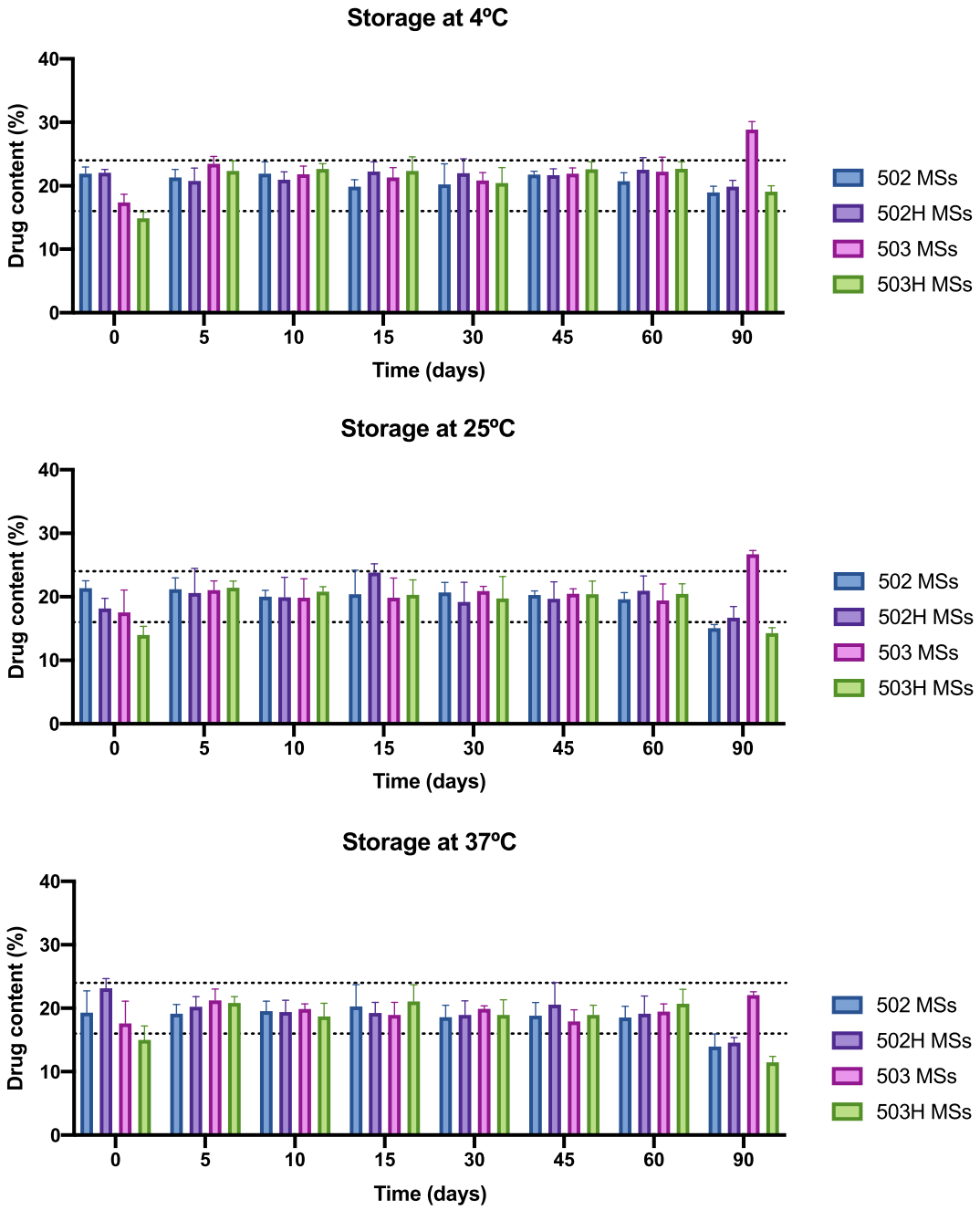

Fig. 6. Resulting data of the stability to storage study at three different temperature conditions for the tested MSs formulations.

Therefore, it has been possible to obtain idebenone-loaded microparticles with a sustained and prolonged release, allowing the achievement of static and uniform drug levels at the site of action.

4.5. Stability studies

4.5.1. Stability to storage

Previous studies showed that PLGA MSs might be associated with aggregates formation at higher temperatures (De and Robinson, 2004). Nevertheless, no aforementioned behavior was observed in the tested formulations, and stability studies were performed as previously established. The results for the short-term stability study showed that all formulations did not suffer major changes during the studied period (see Fig. 6). Negligible changes during the stability study by drug content uniformity may be linked to interferences from the polymer metabolites. Therefore, the idebenone entrapment into PLGA MSs did not modify the drug's native structure. Besides, orange physical appearance was preserved during the entire assay, as well as positive injectability and syringeability test results, supporting the free-flowing behavior of the formulations.

A two-way ANOVA was additionally submitted, and no statistically significant differences ($p > 0.05$) were found among formulations for the same storage conditions during the studied period. Thus, all formulations were stable over the studied time interval.

4.5.2. Stability to pH

Extreme pH conditions of the media may be generally used to achieve a quicker drug release rate from the microparticulate systems due to changes in the release mechanism involved. Stability-to-pH changes were evaluated through MSs uniformity content by UV–Vis

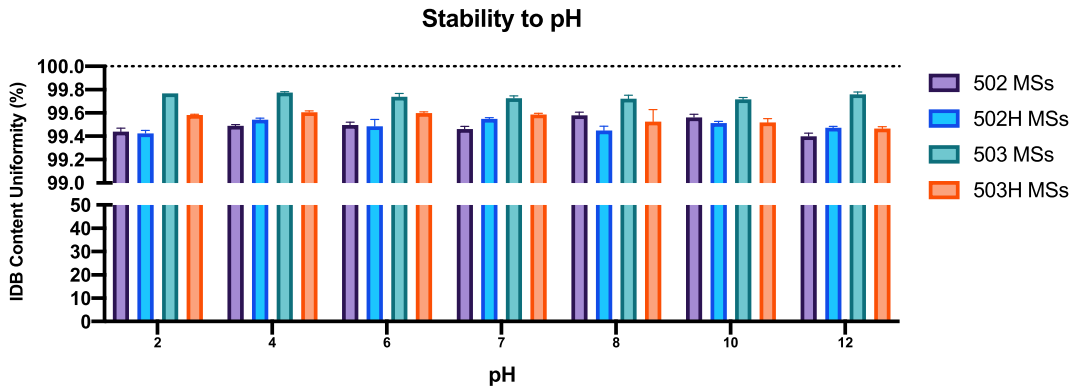

Fig. 7. Idebenone content uniformity (%) values during the stability-to-pH study.

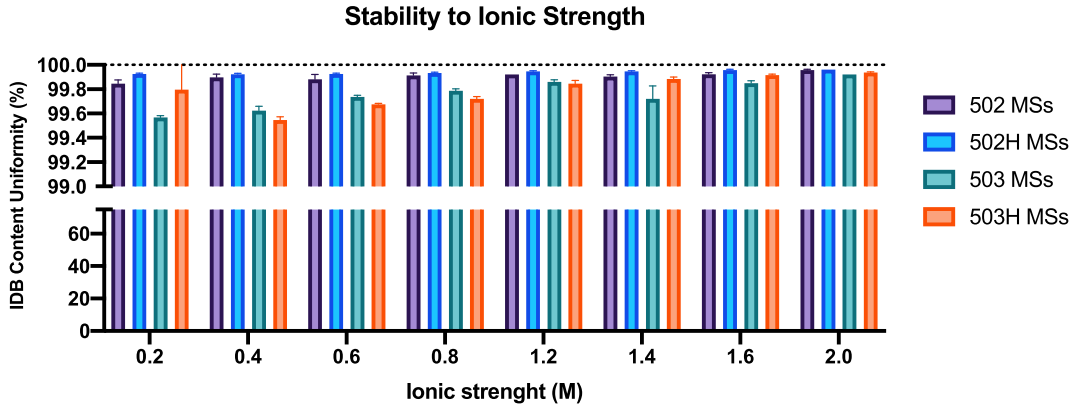

Fig. 8. Idebenone content uniformity (%) values during the stability-to-ionic-strength study.

spectrophotometry, as presented in previous sections. Physical characteristics of the formulations were carefully examined for changes in appearance, color, and clumping/aggregation behavior. The possibility of any MSs collapse or breakage was also assessed by optical microscopy.

Fig. 7 displays the resulting data for the stability-to-pH study of idebenone-loaded PLGA MSs. The hydrophobic nature of the idebenone would prevent its diffusion to the surrounding medium, thus enhancing its entrapment into the PLGA MSs, and protecting the drug from degradation phenomena. In addition, no changes in physical appearance, color or aggregation behavior were observed during the study.

A two-way ANOVA was also applied, and no statistically significant differences ($p > 0.05$) were observed along the studied period. Thus, all formulations were stable against pH variations over the studied time interval.

4.5.3. *Stability to ionic strength*

Extreme ionic strength conditions of the media may be usually used to achieve a quicker drug release rate from the microparticulate systems due to changes in the release mechanism involved. Stability-to-ionic-strength changes were evaluated through MSs uniformity content by UV–Vis spectrophotometry, as presented in previous sections. Physical characteristics of the formulations were carefully examined for changes in appearance, color, and clumping/aggregation behavior. The possibility of any MSs collapse or breakage was also assessed by optical microscopy.

Fig. 8 displays the resulting data for the stability-to-ionic-strength study of idebenone-loaded PLGA MSs. The stability of idebenone-loaded MSs suspension was maintained constant regardless of the ionic strength increase in the media. The hydrophobic nature of the idebenone would prevent its diffusion to the surrounding medium, thus improving its entrapment into the PLGA MSs and protecting the drug from degradation phenomena.

A two-way ANOVA was then submitted, and no statistically significant differences ($p > 0.05$) were found along the studied interval. Thus, all formulations were stable against ionic strength variations over the studied time interval.

Table 8
Data of the moisture content (%) for the selected MSs formulations.

Formulation	Moisture content (%)
502 MSs	4.89 ± 0.91
502H MSs	4.02 ± 1.47
503 MSs	3.97 ± 2.21
503H MSs	4.49 ± 1.11

4.6. *Moisture content*

Moisture content assay may assess MSs stability due to the fact that high moisture content may lead to particle agglomeration phenomena, lessening MSs stability and increasing interparticle cohesion and degradation processes of the formulation components (Zhang et al., 2007; El-Sherbiny and Smyth, 2012). The moisture content of MSs should be under 10% weight of the entire formulation to guarantee the drug stability over time (Sander et al., 2013).

In the present assay, idebenone-loaded PLGA MSs moisture content ranged from 3.97 ± 2.21% to 4.89 ± 0.91%, lower enough to assure formulation long-term stability, avoiding drug degradation (see Table 8). Furthermore, results may correlate with polymer properties, where the more hydrophilic it is, the more water content it will have.

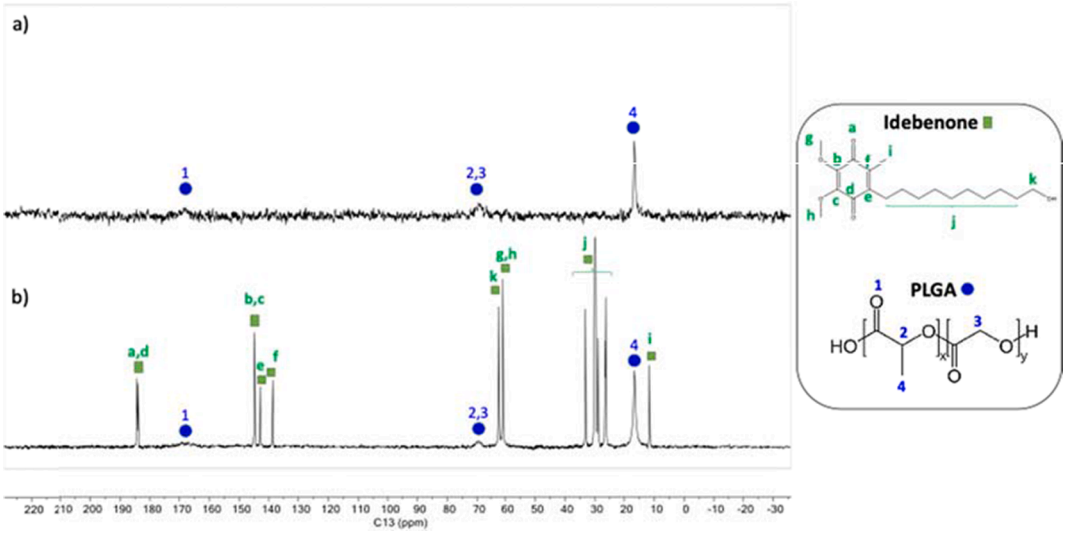

Fig. 9. Solid NMR spectra of formulation 503 MSs. a) 1D ^{13}C-CPMAS and b) 1D ^{13}C-PARISxy. The structures are shown on the right with the letters and number codes used to identify the atoms.

4.7. Solid-state nuclear magnetic resonance (ss-NMR)

Nuclear Magnetic Resonance Spectroscopy (NMR) allows the study of almost any element (isotope) of the periodic table. Molecular information is obtained by means of NMR spectra. There is a huge variety of types of NMR spectra and each of them is sensitive to one type of molecular information (covalent or chemical bond structure, spatial proximity between atoms, molecular translational scattering, rotational scattering, …).

Solid state NMR allows the study of physical and chemical properties of samples in crystalline or amorphous state. Applications include the determination of molecular structure, intermolecular packing in amorphous solids, material properties (e.g., flexibility, fragility, …), the evolution of materials and the study of heterogeneous samples.

Phase separation and heterogeneity in solid formulations of polymers and drugs are relevant for their applications, especially as drug delivery systems (Duan et al., 2020). Even in formulations prepared with the same composition, subtle effects of the protocol of preparation, viscosity, pH or molecular weight among others that may lead to differences in crystallinity as well as the formation of heterogeneous phases with differences in the molecular mobility of the components.

Phase separation and molecular mobility can be studied by ss-NMR methods (Duan et al., 2020; Courtier-Murias et al., 2012; Nasu et al., 2013). In this work, phase separation and mobility of the idebenone-loaded PLGA microparticles were studied by the comparison of two different types of ss-NMR ^{13}C spectra. The former is based on the direct-

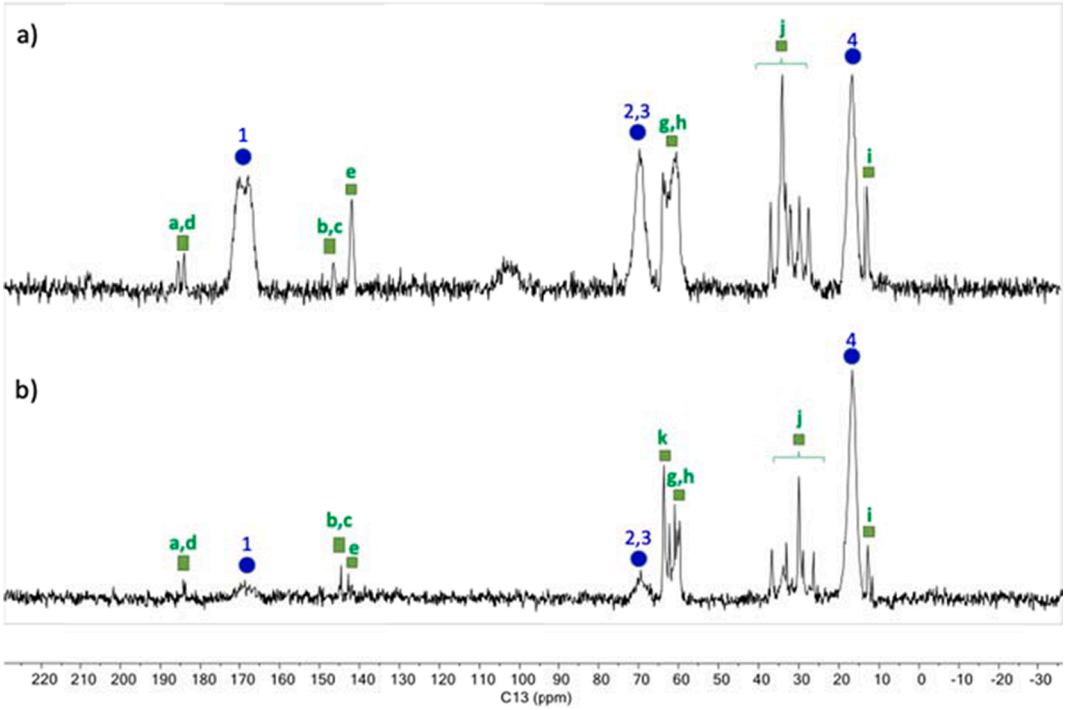

Fig. 10. Solid NMR spectra of formulation 502H MSs. a) 1D ^{13}C-CPMAS and b) 1D ^{13}C-PARISxy. The letters and number codes used to identify the atoms are given in Fig. 9.

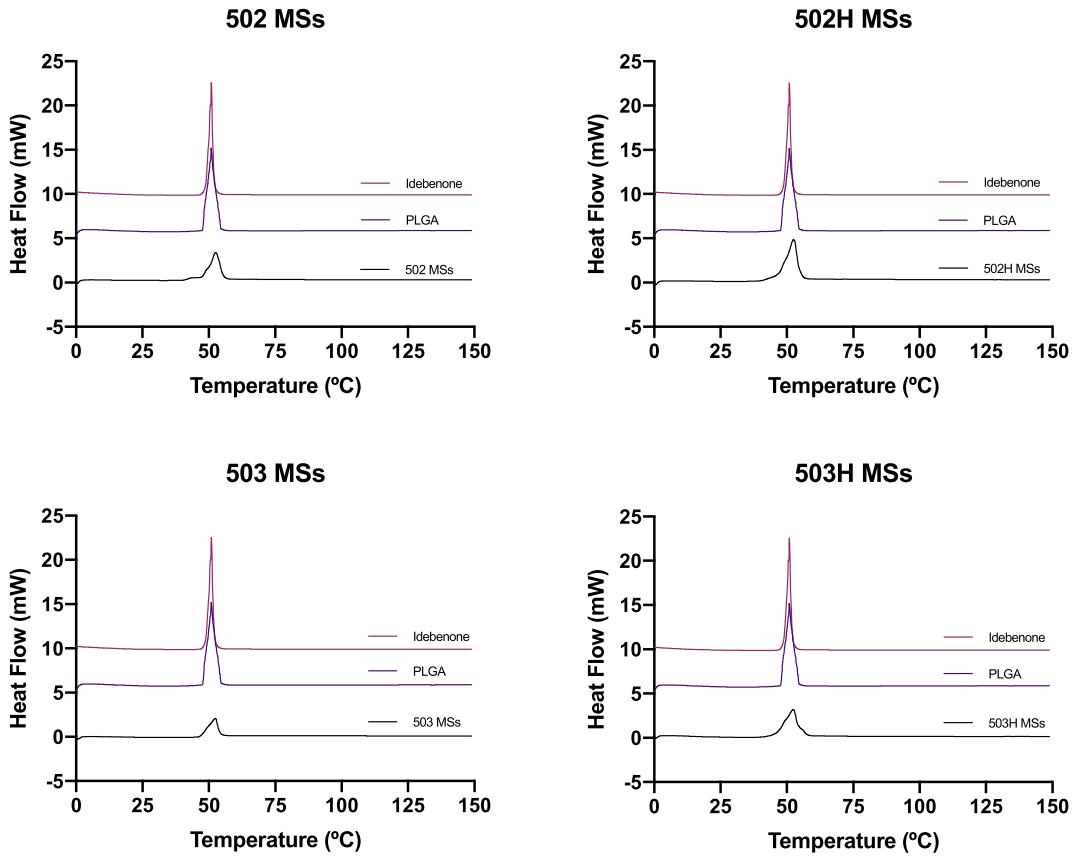

Fig. 11. Differential Scanning Calorimetry (DSC) traces of idebenone-loaded PLGA MSs.

polarization ^{13}C-PARISxy spectrum (Purusottam et al., 2013), while the second one is grounded in the ^{13}C-CPMAS spectrum, related to the cross-polarization phenomenon (Fu et al., 2004; Lange et al., 2009). Both types of ss-NMR ^{13}C spectra have shown high sensitivity to sample heterogeneity and phase formation due to the fact that the signal intensity is affected by the degree of molecular flexibility/rigidity that experiences each carbon site.

The ^{13}C-PARISxy spectrum emphasizes the ^{13}C resonances intensity in a semi-fluid phase characterized by fast molecular mobility and long proton transversal relaxation times. The ^{13}C-CPMAS-spectrum signals are generated by a ^{1}H–^{13}C dipolar interaction during the cross-polarization period of the experiment (Fu et al., 2004; Lange et al., 2009). Since the dipolar interaction is attenuated or vanished for molecules that experience fast liquid-like molecular mobility, the ^{13}C-CPMAS emphasizes signals in a rigid environment characterized by relative short transversal relaxation times. Based in these principles, the comparison of ^{3}C-CPMAS and ^{13}C-PARISxy spectra of the solid formulations was employed to characterize the microparticles in terms of the formation of heterogeneous phases and differences of molecular mobility.

The ^{13}C-CPMAS and ^{13}C-PARISxy spectra of the 503 MSs formulation are shown in Fig. 9. The PLGA component is the only visible in the ^{13}C-CPMAS spectrum of Fig. 9a and its signals appear with considerable broadness, a typical condition of a rigid but disordered polymorphic state. Interestingly, the situation is the opposite in the ^{13}C-PARIS-xy spectrum (Fig. 9b); the signals of idebenone dominate the spectrum and appear as narrow peaks more typical of a fluid or semi-liquid state while the PLGA peaks are barely observed in Fig. 9b despite the fact that is the main component in this sample. Overall, these results denote a different phase for PLGA and idebenone in this sample, the later component showing high mobility entrapped into the PLGA nanocavities. The analysis of the ^{13}C-CPMAS and ^{13}C-PARISxy spectra of the 502 MSs and 503H formulations (see supplementary Figs. 1 and 2) has reached similar conclusions regarding sample heterogeneity as those seen above for the 503 MSs formulation.

The ^{13}C-CPMAS and ^{13}C-PARIS spectrum of formulation 502H MSs are given in Fig. 10 and represents a different situation compared to the other three formulations. The ^{13}C-CPMAS spectrum (see Fig. 10a) shows the idebenone and PLGA signals with good signal:noise ratio. In concordance, the intensity of the idebenone signals in the ^{13}C-PARISxy spectrum Fig. 10b is substantially diminished compared to the analogue spectrum of 503 MSs formulation (see Fig. 9b). Overall, these results denote that PLGA and idebenone in the 502H MSs formulation are both in a rather homogeneous and relatively more rigid environment compared to the other three formulations. Besides that, the high mobility of idebenone in the polymeric environment detected for the other formulations has been considerably reduced.

The ss-NMR results obtained for the four microparticle formulations are in good agreement with those obtained herein by the X-ray diffractometry, DSC and TGA analyses that showed the idebenone amorphization in the MSs formulations. Ss-NMR also provided positive information related to the phase heterogeneity in the polymeric microparticles, in particular for 502 MSs, 503 MSs and 503H MSs formulations and, in a lower extension, for the 502H MSs formulation.

Additionally to the 3-month storage study, the 502, 503, and 503H MSs (1:10 polymer: drug ratio) were stored 12 months in a cabinet at room temperature (17–25 °C) in the absence of light. The 13C-CPMAS and 13C-PARIS spectrum of formulations (supplementary data 3 and 4) do not show significant changes after the 12 storage period indicating that no major changes have occurred in the solid structure.

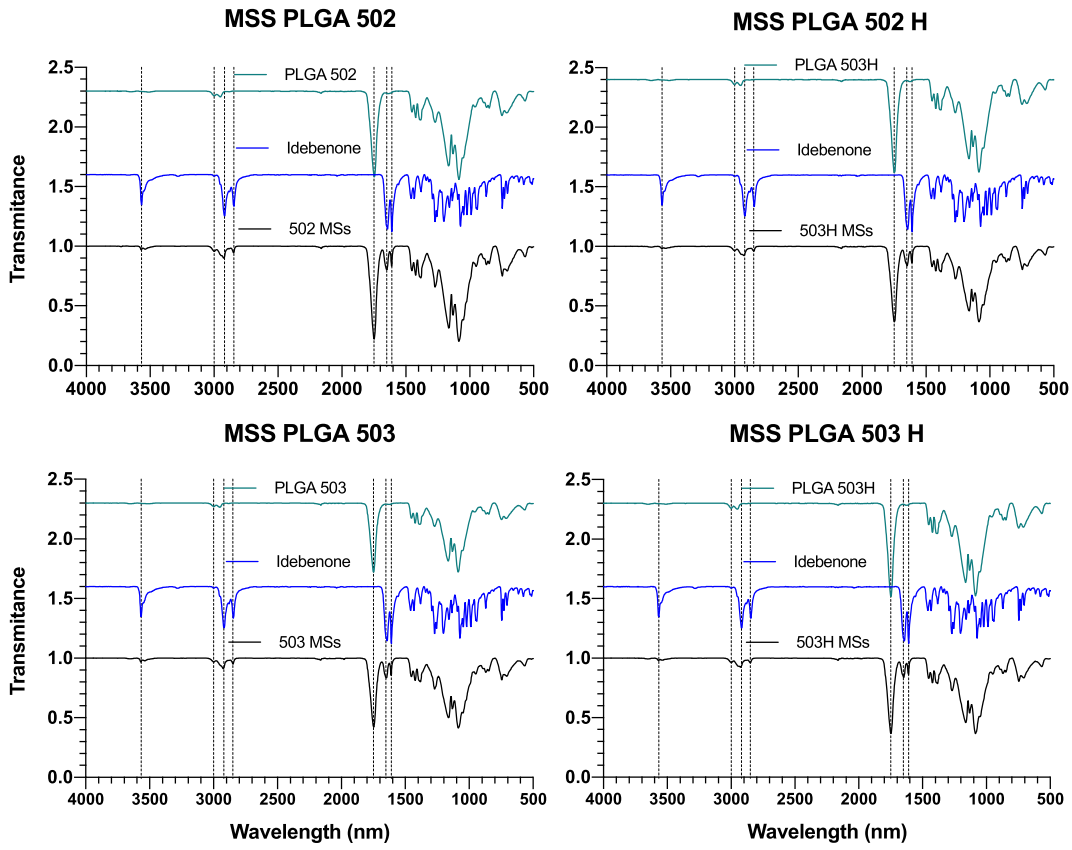

Fig. 12. FTIR results for the components and the selected MSs formulations. Two different transformations ($Y = 2Y_0 + 1$ and $Y = 4Y_0 + 1$) were respectively applied on PLGA and idebenone graph representations to promote the complete observation and comparison of the resulting data.

4.8. Differential scanning calorimetry (DSC) and thermogravimetry (TGA) analysis

DSC curves of idebenone-loaded PLGA MSs were measured to determine the existence of interactions, if any, between the PLGA and the idebenone within the polymer network of the MSs (see Fig. 11).

The DSC curves of idebenone-loaded MSs showed a melting point similar to the transition temperature of PLGA polymers (42–46 °C). No detectable melting endotherm pattern was found for idebenone, possibly due possibly due to a masking phenomenon, by exhibiting a melting temperature similar to that of polymers (52 – 55 °C). Furthermore, no detection was found in terms of drug state into the PLGA-based microspheres based on endothermal point measurement, where molecular dispersion or solid solution states might not be distinguished from the resulting data. Thus, results suggest that both idebenone and PLGA maintained their characteristics into the final MSs formulations. From the MSs thermal analysis, it can be concluded that the idebenone existed in amorphous or disordered crystalline form into the PLGA polymer matrix after the MSs preparation, which may have a noticeable therapeutic importance as it could lead to an enhanced biological activity.

The analysis of TGA/IR results of microspheres showed that weigh changes were not produced during the temperature ramp and no dichloromethane residues were detected by the IR analyzer associated with the TGA equipment. Consequently, no residues of the solvent used in microsphere fabrication was detected.

4.9. Fourier-transformed infrared spectroscopy (FTIR)

FTIR spectroscopy analysis was used to confirm the absence of no chemical interaction between the drug and the polymer. The FTIR spectra of idebenone, the different PLGA polymers and idebenone-loaded PLGA MSs are shown in Fig. 12. Idebenone show their main bands at 3568, 2921, 2846, 1650 and 1610 cm^{-1}, corresponding to the O-H, C-H, C=O and C=C stretching vibrations. PLGA samples show their major bands at 2900–3000 and 1752 cm^{-1}, agreeing with the C-H and C=O stretching bands. FTIR spectra of the MSs samples confirm the absence of chemical interactions among the components of the formulation whereby confirming the chemical compatibility between the drug and the polymer by maintaining the integrity of main peaks. Idebenone characteristic peaks indicate no major shifts, matching the formulation spectrum. Thus, the resulting spectra demonstrate the compatibility between the idebenone and all PLGA tested polymers.

FTIR spectra of formulations 502, 503, and 503H MSs (supplementary data 5) after the 12 storage period do not show significant changes in the main bands of the drug and polymer. These results indicate that no changes in the chemical structure of drug or polymer have occurred, maintaining the compatibility between the idebenone and PLGA throughout storage.

4.10. X-Ray diffractometry analysis

All idebenone-loaded MSs formulations were characterized by X-ray diffraction studies. The physical state of the MSs components was also determined before the preparation of the formulations. The X-ray diffractograms for the MSs components and final formulations are shown in Fig. 13.

The analysis by X-ray diffraction demonstrated the presence of many diffraction bands in the drug's powder sample, as well as for PLGA polymers, reflecting their characteristic crystalline properties. Idebenone remains in its amorphous state, randomly and rather homogenously distributed throughout the PLGA matrix, possibly due to low drug loading in the MSs.

After 12 months of storage at ambient temperature (see supplementary data 6), peaks corresponding to idebenone crystalline are

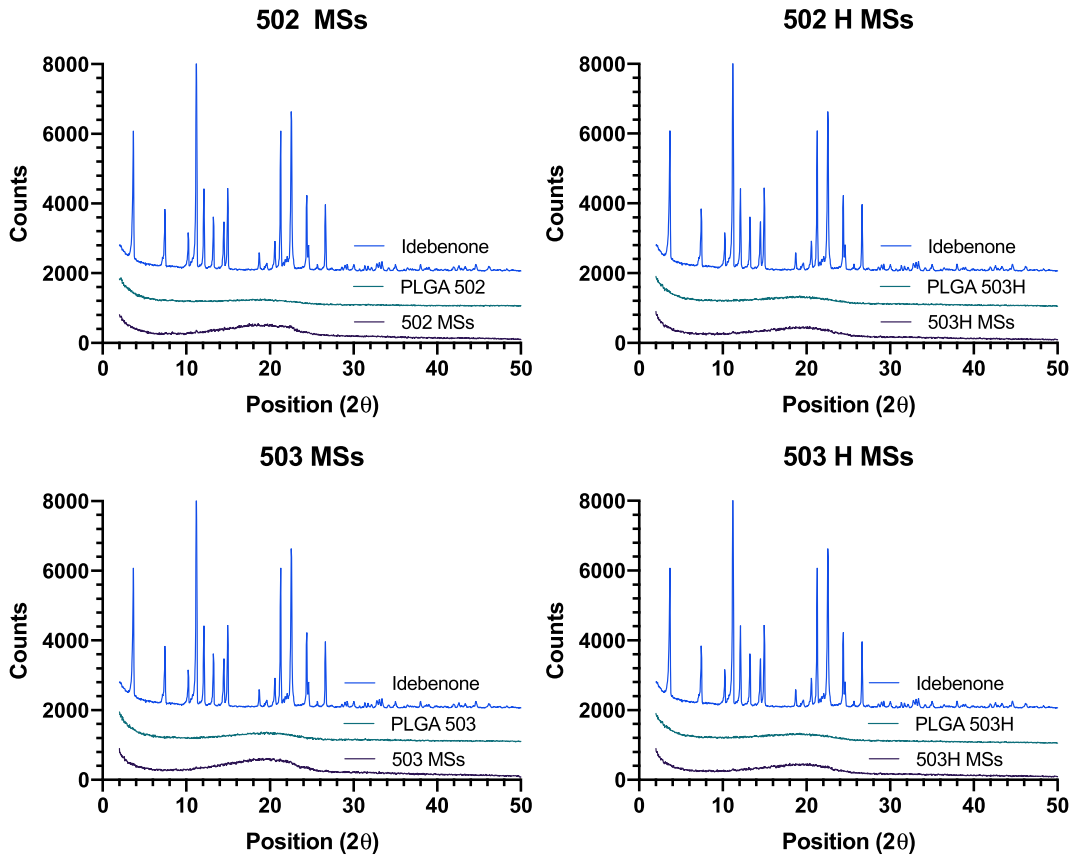

Fig. 13. X-Ray diffractometry results for the components and the selected MSs formulations. Two different transformations ($Y = 1000Y_0 + 1$ and $Y = 2000Y_0 + 1$) were respectively applied on PLGA and idebenone graph representations to promote the complete observation and comparison of the resulting data.

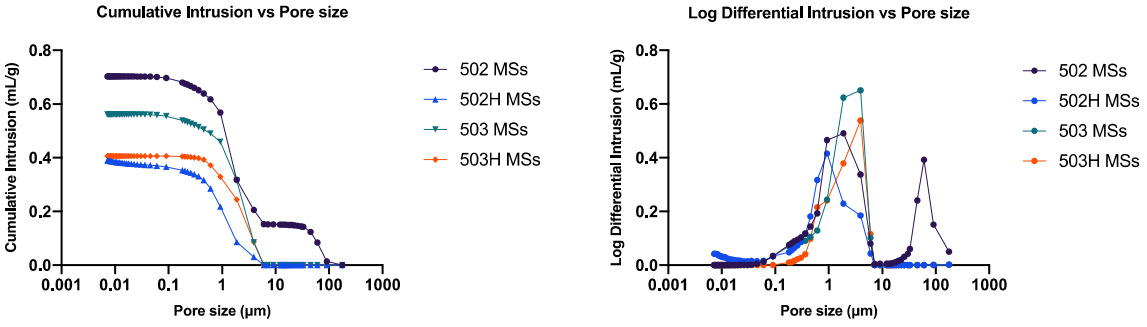

Fig. 14. Resulting data of mercury intrusion porosimetry studies for the different idebenone-loaded MSs formulations. All data were fitted to a log-normal distribution.

observed in 502 MSs samples indicating that a small proportion of the drug is recrystallizing in the MSs matrix. On the contrary, no changes in the amorphous structure of IDB were observed in 503 and 503H MSs.

The differences observed can be attributed to the different molecular weights of the polymers. Resomer® 502 has a molecular weight distribution between 7000 and 17000 Da and Resomer® 503 and 503H between 21000 and 38000 Da. Probably, caused by the low molecular weight, Resomer® 502 provides a less compact and viscous MSs matrix that allows the drug diffusion and migration, allowing the formation of crystallization nuclei. The higher molecular weight of Resomer® 503 and 503H hinders the drug diffusion and migration, inhibiting the crystalline growth of the drug.

The X-ray and NMR results indicate that these MSs have been shown to maintain their stable structure throughout the storage period.

4.11. Mercury intrusion porosimetry

Mercury intrusion porosimetry allows the empty spaces quantification among MSs (inter-MSs porous volume), as well as the pore volume in the MSs microstructure (intra-MSs porous volume). Pore volume distribution of the idebenone-loaded PLGA MSs is shown in Fig. 14. As observed, the cumulative pore distribution of 502H MSs shows the presence of a significant pore population ranging from 5 to 100 μm, representing the 20% of the pore population; hence, this interparticular space value may be associated with the formation of vaults and voids caused by the particle aggregation of the in the porosimeter probe. A main pore distribution with a 1–5 μm mean size is also observed in all MSs formulations, representing the common interparticular spaces. No significant pore distribution was observed for lower values, suggesting that all microparticulate formulations showed low microporosity.

Table 9
Specifications of the different needles used during the syringeability and injectability assays.

Needle gauge	Inner diameter (mm)	Length (mm)
18	1.020	38
21	0.710	32
27	0.361	13
30	0.250	10

4.12. Syringeability and injectability studies

Syringeability and injectability of MSs formulations are key-product parameters of intraocular DDS, where a formulation should be delivered by a syringe and easily pass through a needle. Since it is well recognized that kinematic viscosity of a formulation deeply influences its ejection from the syringe/needle system, the syringeability and injectability of the resulting MSs formulations were automatically evaluated by a panel test created with a universal testing machine. In addition, as the needle gauge might influence the patient's comfort and compliance, different needle consistency was also assessed (see Table 9 for needle specifications).

All predefined subsets were favorable in terms of MSs formulation's injection into air, independently of needle diameter or length (see Table 9). The ease of injection into air was acceptable for the formulations tested. The results of injectability and syringeability revealed neither partial nor complete blockage of the MSs suspension flow (see details in Fig. 15). Thus, resulting MSs are appropriate for intraocular

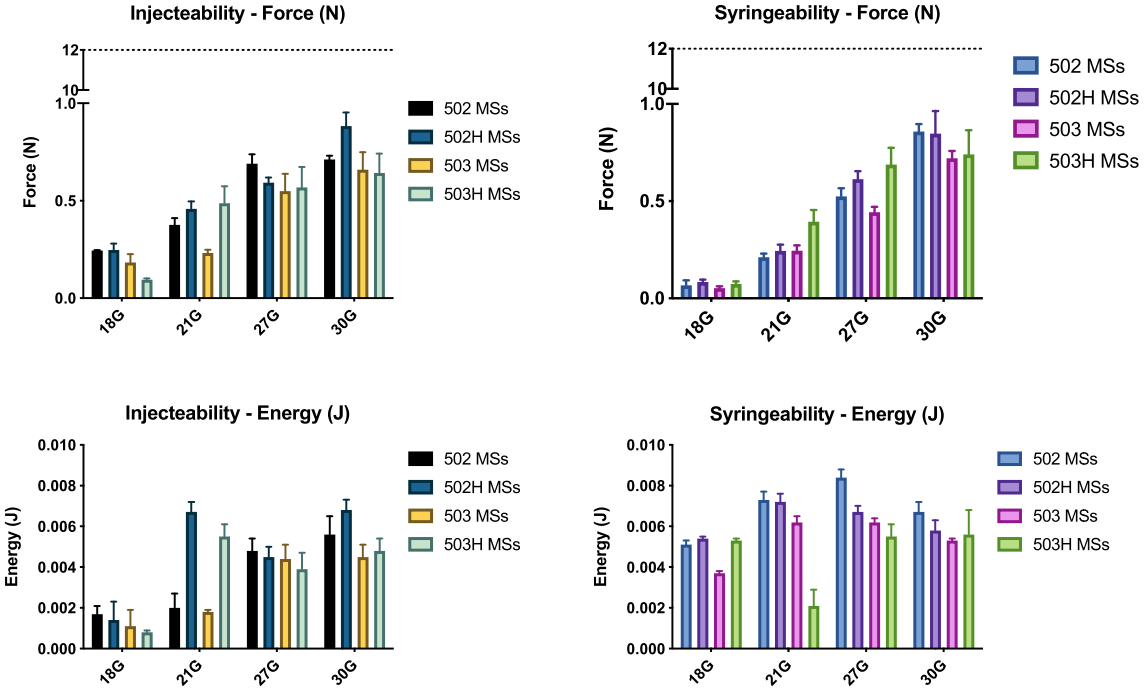

Fig. 15. Injectability and syringeability results for the selected MSs formulations.

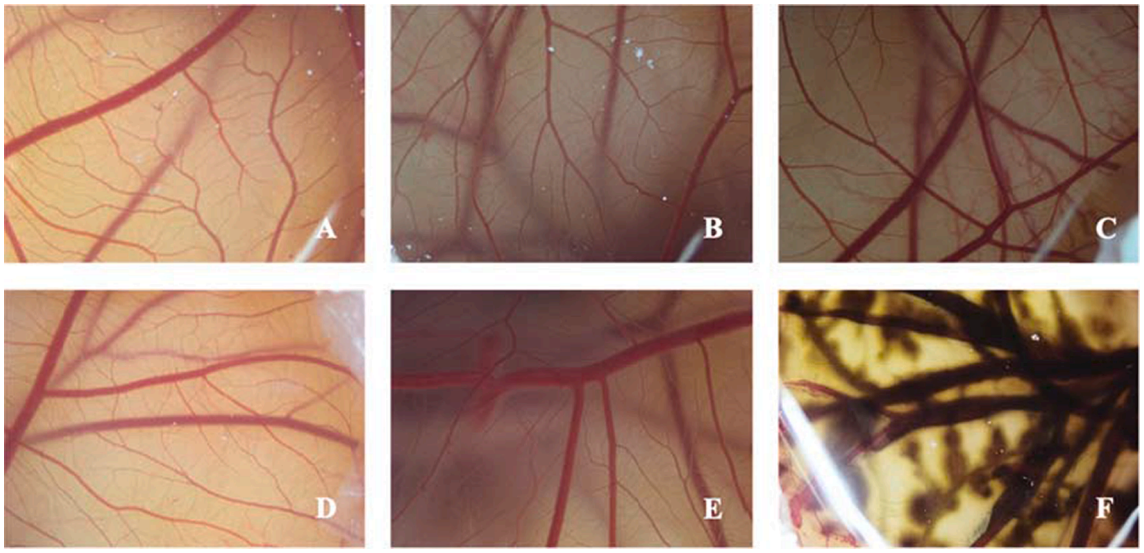

Fig. 16. HET-CAM results for the idebenone-loaded PLGA MSs formulations. (A) 502 MSs, (B) 502H MSs, (C) 503 MSs, (D) 503H MSs, (E) PBS (negative control), and (F) Ethanol (positive control).

injection through a 30G needle.

4.13. Ocular irritancy and toxicity analysis

The CAM is a no innervated complete tissue containing a complex structure of blood vessels (arteries, veins, and capillaries), being a well-developed vascularization organotypic model and an easy-to-study alternative strategy for ocular irritancy and toxicity since it responds to damage with a complete inflammatory process, similar to that produced in the retinal tissue.

Idebenone-loaded MSs cytotoxicity was assessed, and results were compared with those obtained for the negative (PBS) and positive (ethanol) control formulations, respectively. All MSs formulations demonstrate no cytotoxicity effects (Irritation Score = 0), comparing them with control formulations (see Fig. 16). Nevertheless, it will be necessary to perform future studies in animal models to evaluate the long-term toxicity of the MSs formulations, since these systems are intended for long periods of time into the vitreous chamber.

5. Conclusion

In the present work, different idebenone-loaded PLGA MSs formulations were proposed as the first pharmacological alternative based on microparticulate systems for the LHON treatment intended for intravitreal administration. This was reinforced by wide *in vitro* and ex vivo characterization studies. A preclinical coherent basis was accomplished as an alternative to LHON treatment, a pathology with no therapeutic alternatives so far. Developed MSs have shown a smooth, regular, and spherical shape with the optimal particle size interval for intravitreal administration through a 30G needle. Also, as is demonstrated in this work idebenone-loaded PLGA MSs have the advantage of their high capacity to encapsulate high amounts of drug without significantly altering their morphology or their ability to incorporate idebenone. Additionally, a controlled and sustained idebenone release was achieved from all MSs formulations, confirming their use as potential DDS for long-term intravitreal administration of this drug.

The versatility of these systems would enable the inclusion of new drugs intended for intravitreal administration according to the patient's needs and characteristics, as a new method of partial personalization treatment strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2022.121504.

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