Optimising the controlled release of dexamethasone from a new generation of PLGA-based microspheres intended for intravitreal administration

J. Rodríguez Villanueva, I. Bravo-Osuna, R. Herrero-Vanrell, I.T. Molina Martínez, M. Guzmán Navarro

PII: S0928-0987(16)30065-3
DOI: doi: 10.1016/j.ejps.2016.03.012
Reference: PHASCI 3512

To appear in:

Received date: 18 January 2016
Revised date: 3 March 2016
Accepted date: 10 March 2016

Please cite this article as: Villanueva, J. Rodríguez, Bravo-Osuna, I., Herrero-Vanrell, R., Martínez, I.T. Molina, Guzmán Navarro, M., Optimising the controlled release of dexamethasone from a new generation of PLGA-based microspheres intended for intravitreal administration, (2016), doi: 10.1016/j.ejps.2016.03.012

Optimising the controlled release of dexamethasone from a new generation of PLGA-based microspheres intended for intravitreal administration.

Rodríguez Villanueva J.[1], Bravo-Osuna I.[2], Herrero-Vanrell R.[2], Molina Martínez I.T.[2], Guzmán Navarro, M.[1]

[1]Departamento de Ciencias Biomédicas, Unidad docente de Farmacia y Tecnología Farmacéutica, Universidad de Alcalá. Ctra. Madrid-Barcelona (A2) Km. 33,600 28871 Alcalá de Henares (SPAIN).

[2]Departamento de Farmacia y Tecnología Farmacéutica, Universidad Complutense de Madrid. Plaza de Ramón y Cajal 28040 Madrid (SPAIN).

Successful therapy for chronic diseases affecting the posterior segment of the eye requires sustained drug concentrations at the site of action for extended periods of time. To achieve this, it is necessary to use high systemic doses or frequent intraocular injections, both associated with serious adverse effects. In order to avoid these complications and improve patient`s quality of life, an experimental study has been conducted on the preparation of a new generation of biodegradable poly D-L(lactide-co-glycolide) (50:50) (PLGA) polymer microspheres (MSs) loaded with Dxm, vitamin E and/or human serum albumin (HSA). Particles were prepared according to a S/O/W encapsulation method and the 20-40µm fraction was selected. This narrow size distribution is suitable for minimally invasive intravitreal injection by small calibre needles.

Characterisation of the MSs showed high Dxm loading and encapsulation efficiency (> 90%) without a strong interaction with the polymer matrix, as revealed by DSC analysis. MSs drug release studies indicated a small burst effect (lower than 5%) during the first five hours and subsequently, drug release was sustained for at least 30 days, led by diffusion and erosion mechanisms. Dxm release rate was modulated when solid state HSA was incorporated into MSs formulation. SDS-PAGE analysis showed that the protein maintained its integrity during the encapsulation process, as well as for the release study. MSs presented good tolerance and lack of cytotoxicity in macrophages and HeLa cultured cells. After 12 months of storage under standard refrigerated conditions (4±1ºC), MSs retained appropriate physical and chemical properties and analogous drug release kinetics. Therefore, we conclude that these microspheres are promising pharmaceutical systems for intraocular administration, allowing controlled release of the drug.

Keywords: Intraocular administration; Microspheres; PLGA; dexamethasone; human serum albumin; controlled release; stability

1. Introduction

Diseases affecting the posterior segment of the eye are devastating and cause blindness in many patients. In recent decades, highly developed countries have witnessed a dramatic increase in the incidence of such pathologies, especially in those which are age-related (Anand 2014). These include uveitis (Miserocchi 2013), proliferative diabetic retinopathy (Sapieha 2010), macular oedema (Zhang 2014) and macular degeneration associated with age (AMD) (van Wijngaarden 2008). All of these diseases affect the posterior segment of the eye (Gaudana 2010), an area with limited drug acess, cause chronic inflammation and require treatment with sustained drug concentrations at the site of action for long periods of time (Barcia 2005).

Among the possible routes for drug delivery at this level (Herrero-Vanrell 2014), the one that has attracted most attention recently is intraocular administration (Yasukawa 2011). This route avoids the adverse effects of systemic administration (Short 2008) and makes it possible to maintain the necessary drug concentration in the posterior segment of the eye (Andres-Guerrero 2015). Several bioerodible and non-bioerodible implants have been developed to be injected or surgically inserted in the eye, but they are not exempt to produce serious inconvenients (endophthalmitis or intraocular inflammation). Nevertheless, one of the most interesting therapeutic tools for the intraocular route is undoubtedly drug encapsulation in microsphere systems, due to its versatility and capacity to maintain physical and chemical properties over time (Garbayo 2008; Yuan 2009; Yao 2011). Dexamethasone (Dxm) is a fluorinated glucocorticoid with anti-inflammatory action which is used as the treatment of choice in diseases that involve ocular inflammation (Sherif 2002). Encapsulation of dexamethasone in microspheres makes also possible to reduce the number of administrations (Behar-Cohen 2013), minimizing the complications associated with repeated injections (Sampat 2010).

An inherent advantage of the MSs versus implants is due to the gauge needle that is required for intravitreal administration of particles. The biodegradable sustained-release dexamethasone intravitreal insert approved by FDA uses an applicator device of 22 gauges (0,7 mm), considerably higher than required for the microspheres that can be delivered as a conventional injection (Barcia 2009). In addition, MSs are good candidates for personalized medicine too, since they allow to administer the suitable drug dose needed to each patient just by adjusting the volume of the microspheres to be injected, according to the therapeutic window, the intravitreal drug pharmacokinetic, the drug payload in MSs and its release kinetic (Herrero-Vanrell 2014) as well as patient needs (Barcia 2009). If drug release kinetic is well known, the precise amount of MSs required by each individual patient can be achieved by adjusting the concentration to be administered (Barcia 2009) Through the development of microspheres loaded solely with dexamethasone, it has been possible to tailor the drug release profile by using mixtures of PLGA polymers (Wang 2014), but the blends play an important role on burst release effect.

One of the strategies employed to increase encapsulation efficiency and prolong drug release time is to include oily substances in MSs formulations (Martinez-Sancho 2003; Barcia 2005). For example, Martínez-Sancho (Martinez-Sancho 2006) included vitamin A in acyclovir-loaded MSs performing a technological function. Besides vitamin A is an antiproliferative agent, this also exerted an adjuvant pharmacological action. Similarly, it has been shown that vitamin E modulates GNDF release from poly (lactic-co-glycolic acid) (PLGA) copolymer microspheres, protects it from degradation and prolongs release time (Checa-Casalengua 2011). In addition, vitamin E is an important natural antioxidant that exerts an anti-proliferative and suppressant effect on protein kinase C, which modulates glutamate transporter activity and could be of great interest in the prevention and treatment of eye diseases (Engin 2009).

Another important aspect to consider in the development of MSs systems is the integrity of the encapsulated drug, especially if it is protein-related. For instance, albumin has been found to exert a stabilising effect during encapsulation of some peptides and neurotrophic factors (He 2006; He et al. 2006; Rafi 2010) when MSs preparation procedures are based on the double emulsion method (W/O/W). In addition, it has been shown that human serum albumin (HSA) has the capacity to reduce the initial burst effect and release rate of erythropoietin, glycoprotein hormone, from 50:50 poly (DL-lactic-co-glycolic acid) microspheres (Yeh 2007).

Based on the above and following on from previous studies by the research team, the present study focused on the preparation, optimisation and physicochemical characterisation of biodegradable PLGA

MSs loaded with dexamethasone, suitable for minimally invasive intravitreal administration with small calibre needles. Vitamin E and/or HSA in solid state were incorporated into the formulation in order to improve its properties and achieve sustained drug release. HSA was also used as a model to assess the feasibility of this platform for encapsulating proteins without altering their integrity. Furthermore, in vitro studies were conducted in order to evaluate MSs cytotoxicity and MSs stability after 12 months of storage.

2. Materials and Methods

2.1 Materials

Poly D-L(lactide-co-glycolide) 50:50 (PLGA) copolymer, Mw 35,000 g/mol and inherent viscosity 0.32 dL/g (Resomer RG 503®), was purchased from Boehringer Ingelheim (Germany). Polyvinyl alcohol (PVA 72,000 g/mol) was supplied by Merck KGaA, Germany. Vitamin E (α- tocopherol acetate) in liquid form and dexamethasone (Dxm) base (9-fluoro-11b, 17, 21-trihydroxy-16a-methylpregna-1, 4 diene-3, 20-dione) were purchased from Sigma-Aldrich (Spain). Human serum albumin fluorescein isothiocyanate-conjugate (FITC-HSA) was purchased from Tebu-bio (Spain). Dxm raw material was ground in a mortar to obtain a powder with a smaller particle size (6.07 ± 0.23 µm). Dichloromethane, acetonitrile and methanol solvents were obtained from Scharlab (Spain). A Bio-Rad Protein Assay® kit and TGX Stain - Free™ FastCast™ acrylamide solutions were acquired from BIO-RAD Lab., California. Sterile well cell culture plates supplied by Cultex S.L.U. (Spain) were employed for cell cytotoxicity and viability assays. Macrophages were cultured in RPMI medium 1640 supplemented with L-glutamine (2 mM), foetal bovine serum (10%), penicillin-streptomycin (2%) and gentamicin (0.4%), provided by Difco (New Jersey), and L-arginine (1 mM) supplied by Sigma-Aldrich (USA). HeLa cells were cultured in DMEM medium supplemented with foetal bovine serum (10%), penicillin-streptomycin (2%) and amphotericin B (2%), all provided by Sigma-Aldrich (USA). Other reagents were of pharmaceutical or HPLC quality and were used upon receipt.

2.2. Methods

2.2.1. Preparation of Microspheres

Microspheres were prepared using a modified version of the emulsion-solvent extraction/evaporation method previously reported by some members of this research group (S/O/W) (Checa-Casalengua 2011). Three PLGA (50:50) MSs formulations with Dxm were developed, containing the following adjuvants: none (F1), vitamin E (F2) and vitamin E plus FITC-HSA (F3) (Table 1). In all cases, parent Dxm was added to a solution of PLGA in dichloromethane (1:10w/w Dxm/PLGA) to obtain a first S/O dispersion. Then, this S/O system was emulsified at 5000 rpm for 1 min (Polytron® PT 3000, Kinematica AG, Switzerland) with an aqueous PVA solution (2% w/v) to form a dispersed S/O/W system. MSs maturation and consolidation were performed by adding the dispersed S/O/W system to 100 mL of PVA solution (0.1w/v) followed by magnetic stirring for 3 hours at room temperature. MSs were then collected by filtration, frozen at -80°C and freeze-dried (Cryodos® Telstar, Spain). They were subsequently stored until use at 4 ± 1°C in airtight vessels containing silica-gel as a moisture adsorption agent. To prepare F2 MSs formulation, vitamin E was added to the dispersed S/O system formed of a finely ground Dxm suspension in PLGA (1:10 w/w) dichloromethane solution. For F3 MSs formulation, 20 µg of freeze-dried FITC-HSA powder was resuspended in 20 µL of vitamin E by means of gentle sonication (UIS250v, Braun Labsonic, Germany) for 30 s in an ice bath to reduce the risk of protein alteration. Next, this was mixed with the S/O dispersion of powdered dexamethasone and PLGA (1:10) in dichloromethane. Three batches of each type of MSs (F1, F2 and F3 formulations) were prepared as described above. PLGA MSs without Dxm or adjuvants (blank MSs) were also prepared, as a control system.

2.2.2. Microsphere Characterisation

MSs preparation process yield was expressed as the ratio between the amount, by weight, of the MSs obtained and the total amount of all materials used.

MSs particle size and particle distribution were assessed by laser light diffractometry using a Microtrac SRA® (USA) following dispersion of the samples in Milli Q® water. The results corresponded to the volume mean diameter of three independent samples for each formulation. Particle size distribution was expressed in terms of the Span factor (Gavini 2008), determined as:

$$SPAN = \frac{d_{95} - d_{10}}{d_{50}}$$

Where d_{95}, d_{50} and d_{10} are the 95%, 50% and 10% percentile particle sizes, respectively.

The ζ potential was determined using a Zetasizer Nano ZS (Malvern instruments, United Kingdom). Microsphere samples were resuspended in low ionic strength media (isotonic NaCl solution and 5 mM phosphate buffer, pH = 7.4) by means of manual agitation followed by the application of low intensity ultrasound (10 s).

MSs morphology were observed before and after Dxm release assays by means of scanning electron microscopy (SEM, Zeiss DSM 950®). Samples for SEM observation were previously vacuum-dried and surface-coated in a cathode evaporator with 25 nm gold film.
The distribution of the FITC-HSA in the MSs was observed by confocal microscopy. To this, samples were fixed with Prolong® Gold resin (Invitrogen, USA) 24 h previously.

2.2.3. Efficiency of Dxm and FITC-HSA loading and encapsulation in MSs

a) Procedure for extracting dexamethasone from MSs

2 mg of freeze-dried MSs, accurately weighed in Eppendorf® tubes, was subjected to a dissolution-precipitation process to extract Dxm content. Dichloromethane (1 mL) was added to each sample, which was then mechanically stirred for 4 hours in order to dissolve the particle core and extract the drug. Next, the polymer was precipitated with 96° ethanol (0.5 mL) and the suspension was centrifuged (5000 rpm, 10 min, 15°C). The supernatant was collected and filtered through a 0.45 µm cellulose acetate membrane (Sartorius 16555-k®, Barcelona). This procedure was repeated four times and all extractive samples were pooled and freeze-dried to concentrate the Dxm extracted from MSs. Lyophilizates were redissolved in mobile phase and Dxm was quantified by HPLC as described below. The achieved extraction efficiency was 97%.

b) Procedure for extracting human serum albumin (FITC-HSA) from MS

The FITC-HSA encapsulated in F3 MSs was extracted by an independent procedure, in this case employing a liquid-liquid extractive method. A volume of 0.7 mL of dichloromethane was added to 2 mg of MSs, accurately weighed in Eppendorf® tubes, and mechanically stirred for 4 hours to dissolve the polymer. Then, 1 mL of Milli Q water was added and the mixture was stirred for a further 24 h period, followed by centrifugation at 5000 rpm for 10 min at 15°C. The supernatant was removed and another 1 ml of Milli Q water was added. This process was repeated four times. Lastly, all extractive liquids were collected, freeze-dried in low-protein adsorption tubes, and stored in the dark in hermetically sealed containers at 4 ± 1°C. Lyophilizates were redissolved in aqueous tampon medium and FITC-HSA was quantified by spectrofluorimetry as described below. The achieved extraction efficiency was greater than 95%.

c) Dexamethasone and human serum albumin MSs loading

This was expressed as the quantity of drug or protein (M_X) encapsulated by unit mass of microspheres (M_{MS}), and was calculated according to the following equation*:*

$$MSs\ Dxm\ or\ HSA\ Loading\ = \frac{M_X\ (\mu g)}{M_{MS}(mg)}$$

Loading was determined in triplicate for each batch: dexamethasone content was determined in all MS formulations (F1, F2 and F3) while FITC-HSA was only determined in F3.

d) Dexamethasone and human serum albumin encapsulation efficiency (EE%)

This was expressed as a percentage and was calculated using the following expression:

$$EE\% = \frac{Total\ MSs\ Loading\ (mg)}{Theoretical\ Formulation\ Content\ (mg)}\ x\ 100$$

Total MSs Loading is the quantity in milligrams of Dxm or FITC-HSA encapsulated in the total amount of MSs obtained [(Loading µg/mg MS)/1000 x (Weight of MS mg)]. *Theoretical Formulation Content* is the quantity in milligrams of dexamethasone or FITC-HSA used to prepare the MSs.

2.2.4. Analytical Procedures for Dxm and FITC-HAS Quantification

a) Dexamethasone analytical method

Dexamethasone was quantified using the chromatographic method described elsewhere (Barcia 2009). This was adapted and validated to the conditions of this study, being linear in the concentration range between 2.5-10 µg·mL^{-1}. The calibration curve obtained (r^2=0.9986) was;

Peak area = 58966 [Dxm (µg/mL)] - 13568

Limits of detection and quantitation were 0.008 and 0.03 µg·mL^{-1}, respectively. Briefly, the HPLC system consisted of two Beckman system gold 508® pumps, an autosampler and a Beckman system gold 508® UV/VIS detector. Analyses were performed in a 3.9x150 mm W30391M094 C18 5-µm column (Waters Corp., Massachusetts) which was thermostatically maintained at 45°C using a Gecko 2000® heating column system (Amcro GmbH, Germany) and preceded by a 100 mm x 4.0 mm guard cartridge (Waters Corp., Massachusetts). Analysis was performed in gradient conditions with two mobile phases: acetonitrile:water 35:65 v/v (phase A) and pure acetonitrile (phase B), delivered at a flow rate of 1.0 mL/min. The initial mobile phase condition 100%A/0%B was progressively changed to 0%A/100%B from t = 10 min to t = 25 min. Subsequently, initial conditions were recovered in the following five min and ran until the end of the assay (t = 35 min). The injection volume was 100 µL. UV detection of analytes was carried out at 254 nm. None of the MSs components interfered in analysis.

b) FITC-human serum albumin analytical method

An analytical spectrofluorimetric method (Shimadzu RF-540®, Japan) for quantification of FITC-HSA in tampon PBS was performed and validated under the following conditions: 480 and 494 nm for excitation and emission wavelengths, respectively, slit width of 5 nm and signal amplification of 2^7. The calibration curve obtained from standard solutions of FITC-HSA, for concentrations ranging from 0.05 to 0.2 μgmL^{-1}, yielded the following mathematical equation:

$$S_F = 330.33\ [FITC\text{-}HSA\ (\mu g/mL)] - 1.7889$$

Where r^2=0.9883 and the limit of quantitation = 0.025 µg·mL^{-1}.

2.2.5. Differential Scanning Calorimetry (DSC)

Measurements were carried out on a Mettler TC15® DSC (Mettler International, Spain) working in a nitrogen atmosphere (flow rate 20 ml min^{-1}). Samples accurately weighed (4-6 mg) in aluminium crucibles were heated from 0 to 300°C at a heating rate of 10°C/min. Thermograms were obtained of solid raw materials (Resomer 503®, Dxm, and HSA, Dxm/Resomer 503® (1:10) physical mixture) and of unloaded and loaded microspheres (F1, F2 and F3) when freshly prepared and after 12 months' storage. The glass transition temperature (Tg), crystalline melting point (Tm) and normalised enthalpy changes (ΔH) were calculated.

2.2.6 In Vitro Release Studies

These studies were carried out in darkness at 37°C using a thermostatically controlled shaking water bath set to 60 rpm (Clifton NE5®, Nickel-Electro Ltd., UK.). Assays were performed in triplicate for each batch of the three MSs formulations prepared (F1, F2 and F3).

2 mg of freeze-dried MSs, accurately weighed in low-binding Eppendorf® tubes, was suspended in 2 mL of phosphate buffer solution (PBS, pH=7.4) containing 0.02% Tween 80® to facilitate particle dispersion and 0.05% sodium azide as a preservative. The Eppendorf® tubes were maintained in the thermostatically controlled water bath for 30 days. The tubes were centrifuged (5000 rpm, 10 min) every 24 h for the first 3 days, and every 72 h after that, in order to settle the MSs and collect the supernatant. This was used to quantify the Dxm released in each period. The volume of extracted liquid was replaced with fresh PBS buffer solution (pH=7.4) each time a sample was taken. The results of the release assay were then fitted to different kinetic models.

2.2.7 Stability Study

Enough amounts of sample from each batch of the three MSs formulations prepared (F1, F2 and F3) were stored for twelve months under controlled environmental conditions: in airtight containers, protected from light, containing a moisture adsorbent (silica-gel), and maintained at $4 \pm 1^{\circ}$ C. Thereafter, MSs were again observed by microscope, Dxm and HSA content analysed and in vitro release behaviour evaluated.

2.2.8 Integrity of Protein Encapsulated in Formulation F3

The integrity of the FITC-HSA encapsulated in F3 MSs was assessed in freshly prepared MSs and after completing the release assay. Following extraction of albumin from the MSs according to the procedure described above, the protein content in extracts was assayed (Bio-Rad Protein Assay). The extracts were analysed by SDS-PAGE electrophoresis in two types of polyacrylamide gel; at 10% and in a polymer gradient of 4-20%. Certified molecular weight standard protein (10 to 250g/mol) and native FITC-HSA in 50 µL of 8 M urea solution were used as references; sample loaded volume in each gel lane was adjusted to contain 5 µg of protein. Assays were conducted in duplicate.

2.2.9 Cytotoxicity Assays

According with the procedures established in international guidelines and European directives (2004/23/EC 2004), murine macrophage J-774.2 cell line (30,000 cells/well) and a HeLa cell line (20,000 cells/well) were used to test MSs cytotoxicity by evaluating cell viability with the MTT method.

The two cell lines were cultured at 37°C in a humidified atmosphere (95% air and 5% CO_2) in 2 mL wells inoculated with 25 µL of sample (Dxm solutions or suspension of MSs), and incubated for 24 h. Thereafter, the medium was aspirated and 100 µL of MTT solution (0.2 µg/µL) and 0.2% dimethyl sulphoxide (DMSO) was added to cells. After incubation for 1 h, the formazan produced from MTT reduced to mitochondrial cell level was quantified by spectrophotometry (λ = 570nm).

The effect of 1 and 10 µM Dxm solutions, blank MSs and loaded F1, F2 and F3 MSs were evaluated at a particle concentration of 0.25 $\mu g \cdot \mu L^{-1}$. The assay was performed in triplicate for each formulation using the medium as negative control and a 0.005% benzalkonium chloride solution as positive control.

2.2.10 Statistical Analysis

Results were analysed using the Statgraphics® package, version 16.15.1 (Centurion XVI, USA). Statistical significance was determined by ANOVA analysis and differences were considered statistically significant when the probability that the null hypothesis was true was lower than or equal to 0.05 ($p \leq 0.05$). Factors of difference (f1) and similarity (f2) (Shah 1998) were used to compare *in vitro* release assay profiles. Profiles were considered similar when $f1<10$ or $f2>50$.

3. Results

The preparation method employed (S/O/W) yielded smooth surface spherical particles, regardless of their composition, as can be seen in the microphotographs obtained with SEM (Figure 1). Some small surface irregularities were present in formulations containing vitamin E. These were not observed when protein was included in the formulation. The average particle size of all freshly prepared formulations (blank, F1, F2 and F3 MSs) ranged between 25 and 29 μm, with monodispersed and uniform populations and a Span dispersion parameter of around 1.1 units (Table 1). Since most particles were in the range between 20-40 μm ($d_{10} \approx 27$ μm, $d_{95} \approx 45$ μm), the systems were successively filtered through two nylon filters with a pore size of 40 and 20 μm (Gilson Inc. USA) to obtain pharmaceutical systems with a narrow size distribution. After the second filtration, the particles retained on the 20 μm mesh were washed with Milli Q® water to remove excess PVA and then collected and stored in closed containers at 4 ± 1°C for use in subsequent assays. Table 2 shows the results for encapsulation efficiency, mean particle size, ζ potential and drug (Dxm) and protein (FITC-HSA) loading in the selected MSs fraction (20-40 μm), for the four formulations prepared: blank MSs (drug- and protein-free), F1, F2 and F3. Statistical analysis of the results for particle size indicated that the differences between formulations (mean size 26.82 to 27.72 μm) were not significant ($p < 0.05$), and that the population distribution range was also very similar (Span 0.68).

All MSs presented a negative surface charge (between -15 and -20 mV) with this preparation procedure. No statistically significant differences were observed in the ζ potential with respect to the blank MSs (-18.54 ± 1.31 mV), due to their composition (Table 2).

The yield of the selected fraction (size 20 to 40 μm) was lower ($p<0.05$) in MSs formulations F1 (30.8 ± 4.4%) and F2 (30.1 ± 3.7%) than in F3 (41.4 ± 4.0%) and blank MSs (45.9 ± 5.2%). However, differences in encapsulated drug loading were not statistically significant between F1, F2 and F3 (EE ≈ 90%, loading > 80 μg Dxm/mg MSs). Nevertheless, the inclusion of vitamin E in F2 MSs generated a slight decrease in loading (80.9 ± 14.1 μg/mg) with respect to the F1 formulation (90.5 ± 11.3 μg /mg), which only contained Dxm. This reduction was partially offset in the F3 formulation by the presence of solid state FITC-HSA in the MSs (84.9 ± 12.7 μg/mg) (Table 2). On the other hand, the quantity of FITC-HSA encapsulated in the F3 formulation was 0.113 μg HSA/mg MSs, corresponding to an EE of 71.45%. Confocal images (Fig. 2) revealed intense HSA accumulation in the MS matrix, being slight on the surface. It should be borne in mind that FITC-HSA was incorporated in solid state dispersed in α-tocopherol (vitamin E). Values of particle size determined by light scattering techniques are shown in table 1.

The integrity of the protein encapsulated in F3 MSs was assessed using SDS-PAGE. Figure 5 shows the result of the analysis of liquid retrieved after extracting the protein from the MSs according to the procedure described in the materials and methods section. A single band can be seen in each lane, in a position corresponding to the native protein (68g/mol), which suggests that the macromolecule did not fragment.

The aggregation state of both the drug and the materials employed for MSs preparation was analysed by DSC. Figures 3A and 3B show the thermograms obtained for the raw materials, the polymer-drug physical mixture (10:1), blank MSs and F1, F2 and F3 MSs formulations. Table 3 gives the values corresponding to these thermal events. In the case of the parent polymer, 50:50 PLGA (MW 35.000g/mol), a reversible thermodynamic transition occurred at 53.80°C, corresponding to a glass transition (Tg). By contrast, Dxm presented a first-order endothermic transition, with a melting point (Mp) at 262.3°C. This is in agreement with the literature (Rodrigues 2009) and confirms its crystalline structure. In the case of the FITC-HSA protein, no thermal event occurred in the temperature range studied (0 - 300°C), up to carbonisation.

The DSC thermogrames for the physical mixture (PLGA:Dxm) and the three MSs formulations loaded with the drug (F1, F2 and F3) showed two thermal events: one Tg, characteristic of the polymer, and another melting endothermal, due to Dxm. However, when Dxm was encapsulated in the MSs (F1, F2 and F3), the drug's melting point, 215° C, was significantly lower than that obtained for the raw material and the physical mixture. On the other hand, there was a drastic reduction in the enthalpy of fusion of the encapsulated Dxm, which did not occur in the physical mixture. These findings suggest that the MSs preparation process could modify the crystalline characteristics of the drug. Therefore, although Dxm was added in solid form to the polymer solution (powder dispersed in vitamin E), it is to be expected a fraction of the drug was dissolved in dichloromethane and thus incorporated into the MS matrix as a non-crystalline molecular dispersion during MSs maturation and consolidation. In contrast,

due to its high molecular weight, the albumin could partially modify the molecular conformation of the PLGA polymer chains, inducing a reduction in their Tg to 51.5° C.

The release assay results for Dxm-loaded MSs in PBS at 37°C are shown in Figure 4A as the cumulative percentage released, and in Fig. 4B as the average release rate in each period (amount of drug released per unit of time µg Dxm/d).

A global analysis of cumulative release curves indicated that formulations F1 and F2 were similar to each other (similarity factor f2>50), whereas there were differences between these and F3 MSs (difference factor f1<15).

In general, initial drug release from MSs is high and is often attributed to a "burst effect". However, none of the three Dxm-loaded MSs developed in this study showed a high drug release for the first 5 hours. Nevertheless, release was significantly higher ($p<0.05$) from both MSs formulations without albumin, F1 (3.5 ± 0.79%) and F2 (4.73 ± 1.64%), than from F3 MSs (1.36 ± 0.26%), which did contain albumin. On the other hand, the total percentage of Dxm released after 30 days by F3 MSs (18.39 ± 1.82%) was lower than that released by either F1 MSs (42.80 ± 2.96%) or F2 MSs (44.56 ± 1.44%).

In order to determine the release kinetics that best described the process, the data were fitted to various kinetic models, including classic ones (Higuchi, Peppas, Hixon-Crowell, first-order) and other more recent ones (Klose-Siepmann and Gallagher-Corringam). Although high correlation coefficients were obtained with the latter models ($r^2 > 0.9$), a significant deviation was observed at the latest time values.

Regarding the cumulative release profiles, two phases could be clearly distinguished: the first one showed a more rapid release, lasting approximately 7 days, while in the second phase, which lasted until the end of the assay, the release rate was slower. On the basis of this biphasic profile, the drug release kinetics were fitted to a biexponential equation or summatory function, as a result of two diffusion and erosion processes.

$$\frac{\Delta Q_t}{\Delta t} = a \cdot e^{-K_1 \cdot t} + b \cdot e^{-K_2 \cdot t}$$

Where ΔQ_t is the quantity of Dxm released in each incubation period and Δt the interval time period. $\Delta Q_t/\Delta t$ represents the average release rate in the interval time considered (µg Dxm/d). K_2 and K_1 are, respectively, the first-order rate constants that characterise both processes and a and b their coefficients (Table 4).

In the period comprising the first 7 days, the percentage of Dxm released by formulations F1 (35.17 ± 1.95%) and F2 (39. 92 ± 3.99%) was very similar and higher than that of the F3 formulation (14.77 ± 1.67%). In the second phase (7^{th} to 28^{th} day), the average release rates were significantly lower than those in the first 7 days.

A statistical analysis of the values obtained for release rate constants revealed no significant differences in K_1 between the F1, F2 and F3 MSs formulations. However, significative differences were observed in the K_2 constants from F3 when compared with those of F1 or F2 MSs.

After 12 months' storage at 4 ± 1°C, particle size and drug loading remained unchanged. Similarly, no alterations were observed in the integrity of HSA incorporated in formulation F3, as shown by the SDS-PAGE electrophoretic analysis (Figure 5). Such was not the case, however, for the ζ, potential, which was reduced in all formulations, and most markedly in F1 and F2 MSs.

With regard to the release studies conducted after 12 months, MSs behaviour remained similar to that observed in the initial assays, again presenting two phases. The cumulative percentages of Dxm released by the MSs at the end of the assay did not present any statistically significant differences with those obtained for freshly prepared MSs: 44.86 ± 2.78% from F1, 42.72 ± 2.16% from F2 and 22.54 ± 2.07% from F3 (Table 4). The mass balance analysis performed at the end of the assay confirmed the release data. However, release was slightly higher in the first 7 days.

In vitro cytotoxicity results are shown in Figure 6. Cell viability of cultured macrophages (Fig 6A), normalised to control, was higher than 90% in all cases. Moreover, no significant differences ($P > 0.05$) were found with the control (100% viability) when macrophages were incubated for 24 h with dexamethasone solutions (1 and 10 µM) or MSs (blanks, F1, F2 and F3) (Figure 6A). In contrast, HeLa cells cultured with blank, F1 or F2 MSs showed a lower survival percentage ($p < 0.05$) than those cultured with dexamethasone solutions (1 and 10 µM) or F3 MSs (95.27 ± 4.40%). In the case of the latter, no statistically significant differences were observed with control (Figure 6B).

4. Discussion

The controlled release of Dxm for long periods of time has proved to be an appropriate strategy for the treatment of chronic diseases affecting the posterior segment of the eye (Comyn 2013). However, there is still a need for MSs systems that release the drug at an appropriate rate for 3 months, 6 months or whatever time required since no every ocular disease and stage courses with the same inflammation grade (Nita 2014).

The aim of this research was to develop extended-release Dxm formulations for intraocular administration, that allow to adjust the dose to the specific needs required in each case, by using a S/O/W approach and incorporating as adjuvants α-tocopherol, which possesses a dual antioxidant and antiproliferative action, and a protein, FITC-HSA, in order to modulate the release process.

Among other factors, the release rate of a drug encapsulated in MSs depends on the composition and microstructure of the matrix and the surface characteristics, including specific surface area; very porous or very small MSs are unsuitable for the development of extended-release systems. On the other hand, the larger the MSs, the larger the calibre needle required for intravitreal administration, which can lead to problems of obstruction and lesion in the eye. The needle must be of sufficient length to permit full penetration to the pars plana, while at the same time allowing the perforation at the site of the injection to close naturally once the needle is removed. It is therefore recommended that 30 G calibre needles or smaller be used for administration (Avery 2014).

One advantage of MSs is that they can be administered in the vitreous with 23 - 30 G needles (Behar-Cohen 2013). Bertram et al. (Bertram 2010) prepared 15-20 μm PLGA 502H BDNF MSs which could be injected with a 30 G needle at a concentration of 25 mg/mL, while Cho et al. (Cho 2005) demonstrated that MSs of PLGA (75:25) with 52 μm could be injected with 18 to 26 G needles.

Consequently, in order to achieve a balance between those two essential aspects (Rodríguez 2013), one aim of this study was to optimise the release of Dxm using HSA and the S/O/W method developed previously (Checa-Casalengua 2011) with an appopriate particle size. Regardless of their composition, MSs thus obtained were spherical with a smooth surface, and those with a particle size ranging between 20 and 40 μm were selected and stored in a freeze-dried state. A preliminary injectability test (unpublished results) with stored freeze-dried MSs showed that they could easily be redispersed in aqueous salt solutions or phosphate buffer and were viable for intravitreal injection with 30 G needles.

The surface of the MSs prepared with formulations F2 and F3 was smooth with some small pores, related to the inclusion of vitamin E. The addition of vitamin E into the organic phase slowed dichloromethane diffusion outside the droplet towards external aqueous phase, generating small pores on the initial seed during MSs maturation and consolidation. It is this which gives rise to the characteristic "golf ball" morphology of MSs with oily additives (Fig. 1) (Fernandez-Carballido 2006).

The ζ potential exerts an important influence on surface properties, stability (aggregation phenomena) and in vivo behaviour of MSs. The negative charge of the ζ potential obtained confirmed the absence of protein on the MSs surface, since HSA would be protonated at a pH = 7.4 and would therefore be positively charged. The range of the potential in all formulations was between the -10 and -20 mV required to avoid non-specific interactions (Davis 2009).

MSs carried a high load of Dxm (90 μg/mg MS). With the preparation method employed (S/O/W), Dxm was incorporated as a fine powder (6.07 ± 0.23 µm) in the initial dispersed system. Given the solubility parameter value (δ $MSa^{1/2}$) for dichloromethane (δ 20.4), PLGA (δ 20.2), α-tocopherol (δ 20.24) and Dxm (δ 13.93) (Madsen 2015), the interposition of phases gave rise to a heterogeneous dispersed S/O system in which Dxm remained partially dispersed in solid state inside the oily phase formed by dichloromethane, PLGA and α-tocopherol acetate. Due to its low solubility in water (0.035 g/l pH 7) and partition coefficient (log P = 2. 03 $\pm$ 0.6), drug diffusion from the immature MS matrix to the external aqueous phase was very reduced, resulting in a high encapsulation rate.

Similarly, albumin was also incorporated into the formulation in solid state, dispersed in α-tocopherol.

The confocal microscope images of F3 MSs show a greater accumulation of albumin in the matrix when compared to the surface. This is related with the MSs preparation mechanism. When a homogeneous drop of O-phase is placed in the aqueous media, organic phase diffuses into water creating a hardened shell

entrapped protein. In 20-40μm MS the diffusion gradient between the microspheres and the outer water phase, the driving force for albumin diffusion, would not be high enough to distribute HSA near the surface. However, due to their hydrophilic nature, it would be plausible to assume that small amounts of FITC-HSA on the surface of the droplets on the dispersed first system (S/O), can be dissolved and diffuse into the aqueous phase secondly added during MSs preparation (S/O/W). This might explain a lower value of EE (71.45 ± 2.01%) obtained for the protein compared with Dxm (EE > 90%), which was nevertheless high compared with that found by other authors who have used the W/O/W method. In the present study, the lack of an internal aqueous phase strongly hindered diffusion from the S/O system to the external aqueous phase during MSs maturation and hardening, and thus encapsulation efficiency was high.

The burst effect is related to the porosity and quantity of drug adsorbed on the MSs surface. Given the characteristics of the preparation method (S/O/W) and that the final process included filtration and washing, it is logical to assume that only a minimal amount of drug would be present on the surface and outermost layer of the MSs. Huang et al. (Huang 2015) used hard X-ray nano-tomography to study the microstructure and porosity of PLGA MSs prepared with the W/O/W method. They observed a high porosity (19.27 ± 3.42%) in MSs measuring 2.35 ± 0.26 μm, which correlated well with "surface protein interconnectivity" and suggested that the initial explosive release at 5-10 h (burst effect) may be the result of rapid drug diffusion through what these authors refer to as interconnected pores.

The S/O/W method yielded less porous systems than the classic double emulsion (W/O/W) procedure, due to the absence of an internal aqueous phase. This is because some of the water remains trapped within the matrix when the polymer precipitates during maturation. Subsequently, this water is eliminated during MSs drying or freeze-drying, leaving cavities or pores in its place. Klose D et al. (Klose 2006) also demonstrated the influence of microstructure, particle size and porosity on the release of HSA from 50 to 110 μm PLGA MSs prepared by a double emulsion method. In another recent study conducted by Madsen G.C. et al. (Madsen 2015), release of BSA from a PLGA matrix was correlated with intrinsic viscosity (η) and solubility parameters (δ). They found that the closer the respective $\delta_{solvent}$ and $\delta_{polymer}$, the greater the hydrodynamic volume occupied by the polymer and the greater the intermolecular interactions. These interactions increased still further as system viscosity increased, influencing both parameters (η and δ) in the internal microstructure and thus the release rate. On the basis of the above, it may also be assumed that MSs microstructure can be affected by changes in the composition of the formulation and the viscosity of the oily phase of the dispersed S/O/W system. α-Tocopherol is a high viscosity Newtonian liquid (4 Pa.s at 20°C) which could act in this sense, modifying the porosity of the MSs nucleus. However, the F2 and F3 MSs formulations only included a small proportion of α-tocopherol (20 μL) and although this slightly increased the viscosity of PLGA solutions in dichloromethane, this alone was not sufficient to modify the drug release process. Thus, the release curve profiles for F1 and F2 were similar (f2 > 50). In the case of F3 MSs, both, the curve profile (f1<15) and the release percentage at 28 d (22.54 ± 2.07%) were different.

Microspheres loaded with hydrophobic drugs such as dexamethasone usually present three-phase release profiles with an initial phase of immediate release (burst effect), followed by a controlled release phase and then a combination of polymer diffusion and erosion (Wang 2014). The curves obtained in the release study at pH = 7.4 presented a profile typical of the PLGA systems employed. However, as discussed above, no marked burst effect was observed in the present study, but two phases could be clearly distinguished: a first phase of more rapid release, lasting approximately 7 days, followed by another second phase of slower release which lasted until the end of the assay.

Once PLGA MSs come into contact with the incubation medium (PBS buffer, pH 7.4), a imbibition process begins, at a much higher rate than that at which hydrolysis of the ester bonds in the polymer occurs. Thus, release initially occurs by diffusion, as the drug dissolves in the water being imbibed. The dissolution rate depends on the drug aggregation state in the matrix. In view of the DSC results obtained, it can be deduced that some of the Dxm was in crystalline form and some of it was molecularly dispersed in the polymer matrix. It is this molecularly dispersed Dxm that dissolves first during imbibition, and which is released first, by diffusion, into the incubation medium. A kinetic analysis of the release process in the first 7 days indicated that this was an anomalous, non-Fikian, diffusion kinetics since the n values were 0.72 for F1 MSs, 0.71 for F2 MSs and 0.68 for F3 MSs.

Besides diffusion, drug release from PLGA MSs in an aqueous environment occurs as a result of the polymer erosion rate, which depends on system porosity. Erosion occurs in the entire mass of the polymer, and the acids generated when PLGA molecules degrade are responsible for the autocatalytic effect. However, in the present case the incubation medium was a buffer solution with a pH of 7.4, which

was periodically removed and replaced with fresh buffer, and thus no sharp decrease in pH was observed (at least on the exterior of the MSs). As a result, the process of erosion took place slowly.

The morphological assessment carried out by means of SEM throughout the release study supported the hypothesis of a homogeneous hydrolytic degradation starting from the surface, similar to that observed by other authors, which subsequently results in the formation of pores and cavities in the matrix as incubation time extends (Wang 2010). It has been shown that the loss of polymer mass in PLGA MSs is a result of the degradation process, and the drug release kinetics can be described by a first-order kinetics (Dunne 2009). Accordingly, it was postulated that the kinetics of Dxm release from PLGA (50:50) s prepared by means of the S/O/W method could be described using the sum of two first-order kinetic processes: one would represent Dxm release by dissolution/diffusion of the drug accessible to the imbibition liquid that moistens the MSs, and the other would represent the release of the drug trapped in the polymer as this is degraded. Fitting was performed by non-linear regression, yielding a very good correlation (Table 4). The results showed that the strategy designed provided a slow Dxm release rate which could be modulated with the incorporation of α-tocopherol, and above all of protein, and which could sustain drug release for a period of more than 30 days.

The slower release rate observed with F3 MSs could be due to the possible formation of a HSA-Dxm complex in the MSs matrix. Interaction between drugs and plasma proteins is a rapid process (Schmidt 2010). Gonciarz A et al. (Gonciarz 2012) studied the binding between Dxm and HSA in PBS buffer solution (pH 7.4) using capillary electrophoresis/ frontal analysis. In the light of their results, they concluded that there is a strong interaction between both molecules (88% Dxm union) with an intermediate degree of affinity (Scatchard equilibrium association constant Ka (M^{-1}) 1.08 x 10^4).

When MSs were in contact with the dissolution medium, the aqueous imbibition front converged with the protein and the drug as it passed through the MSs. This led to dissolution of both molecules (both dispersed in solid form in the matrix) in this aqueous medium, rendering it possible for them to interact either directly or when the Dxm diffused through the polymer matrix in its release.

On the other hand, due to the difference between the molar volumes of Dxm (296.2 cm^3/mol) and HSA (4.6 x 10^4 cm^3/mol) (Coccoli 2008), it would be reasonable to assume that the drug release rate from the MSs would be lower for protein. Thus, the diffusion and erosion phases presented a slower kinetics than that of the active ingredient in F2 and F3 MSs formulations. In addition, as evidenced by the SEM images (Fig 1), the presence of HSA and vitamin E in the formulation reduced polymer degradation, yielding a more sustained release in the last phase.

The microencapsulation process could generate certain conditions that can give rise to HSA denaturation or fragmentation, which may result in loss of activity (Aubert-Pouessel 2002; Jiang 2007; Giteau 2008). It is therefore necessary to confirm that the protein has retained its integrity once encapsulated. To avoid protein denaturation and the aggregation phenomenon observed in the CH_2Cl_2/H_2O interface (Sah 1999), the encapsulation technique employed was a modified version of the emulsion-solvent extraction/evaporation method previously proposed by some members of this research team (S/O/W) (Checa-Casalengua 2011). Freeze-dried FITC-HSA powder was resuspended in vitamin E and then mixed with the S/O dispersion of powdered dexamethasone and PLGA (1:10) in dichloromethane. This method is based on the idea that when protein is trapped in a given conformation in a particle matrix, changes in its folding are unlikely to occur (Garbayo 2009). Furthermore, compared with S/O/O methods, the modified S/O/W method reduces the quantity of oily phase residues, which can lead to immunological problems in patients and difficulties in obtaining approval from regulatory agencies (Yuan 2009).

The similarity of the SDS-PAGE electropherogram bands shown in Figure 5, corresponding to native protein and aqueous extracts from F3 MSs before and after the release assay, indicates that the FITC-HSA maintained its molecular weight. In addition, the spectrofluorimetry study revealed no change in protein excitation or emission spectra either in freshly prepared MSs or 28 days after drug release studies. Thus, it can be concluded that the procedures used in the encapsulation process (sonication, emulsification by high speed homogenisation in dichloromethane, freeze-drying) did not alter FITC-HSA integrity .

The results also show that storage time did not have any negative effect on the morphological, physical or chemical properties of the MSs, the structure of the encapsulated protein or the release profiles of the incorporated active ingredient, Dxm.

The design of a new MSs system for biomedical purposes requires an understanding of its interactions with biological systems at the cellular level in order to optimise effects on target cells and diminish side effects on healthy ones. Human macrophages (Mφ) and the human epithelial carcinoma cell line (HeLa

cells) have been widely used to investigate the toxicity of different compounds and to explore the rather complex processes involved in interactions with particles (Lee 2012; Fratoddi 2015; Srivastava 2015). There are predicted gene products for a wide variety of HeLa and Mφ extrinsic components and stress conditions (Chovatiya 2014). Most of them mediate apoptosis, thus turning this cell lines excellent as *in vitro* models of toxicity induced by environment changes (Albanese 2011). Both types of cell were used in this study to conduct a preliminary assessment of the cytotoxic effect of PLGA (50:50) MSs for 24 h. Macrophages showed little sensitivity to MSs (cell viability was higher than 90% in all cases), and thus it was not possible to discriminate any differences in cytotoxicity that might exist between them. By contrast, HeLa cell cultures proved to be more sensitive than murine J-774.2 macrophages, showing a slight but significant ($p<0.05$) decrease in cell viability when incubated with drug-free MSs or F1 and F2 MSs. Dexamethasone solutions presented no difference with the control, although a very slight but non-significant reduction in macrophage and HeLa cell viability was observed at a concentration of 10 μg/mL. This negative effect of PLGA MSs on HeLa cells was only mitigated in the F3 formulation (cell viability was higher than 95%).

In mammalian cell culture, fetal bovine serum (FBS) and proteins including albumin (BSA) have been extensively added to culture media as growth factor. A key interaction of HSA with cells involves its antioxidant properties, that are relevant to the extracellular environment of the cell as well as in the intracellular compartment (Halliwell 2004), having the potential to influence a wide range of cell processes (Francis 2010). Among them, HSA promotes cellular survival after apoptosis induction (Schiller 2008), and for endothelial cells exhibits an anti-apoptotic activity via a G coupled PI3 K-dependent mechanism (Bolitho 2007). On this basis, it is logical that free FITC-HSA could increase survival by interacting with cell membrane.

5. Conclusions

An efficient and reproducible method for encapsulating Dxm in biodegradable PLGA (50:50) microspheres has been described. The pharmaceutical system selected (20-40μm) showed appropriate physical and chemical characteristics, yielded controlled drug release for prolonged periods of time and was stable under standard refrigerated storage conditions. A preliminary injectability test has shown that Mss were viable for intravitreal injection with 30 G needles.

The excipients included in the MSs formulation provided stability to the first drug suspension in dichloromethane (vit. E) and contributed to modulating drug release (HSA). In general, MSs presented good biocompatibility (cell viability was above 85% in all cases), with HeLa cells being more sensitive than murine J-774.2 macrophages. F3 MSs, containing Dxm, vitamin E and HSA, showed the best cellular behaviour, similar to dexamethasone solutions. The novel strategy reported is thus a good candidate for further in vivo studies and could be a useful tool in the treatment of chronic degenerative diseases.

Acknowledgements

This research was made possible by funding provided through the "Nanopharmacy" programme of the General Foundation of the University of Alcalá (FGUA) and UCM920415 (GR3/14) project of the Complutense University of Madrid (UCM). Javier Rodríguez thanks the UAH for the award of the AIA 2014 grant, and Dr. Amelia Hernández for her support. The authors also thank the Microscopy and Cell Culture Units of the Research Support Centres at the Complutense University of Madrid (UCM) and the University of Alcalá (UAH).

Conflict of interests

The authors declare no conflict of interest when conducting the study.

Bibliography

2004/23/EC (2004). European Parliament and the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells

Albanese, A., Chan, W. C. (2011). "Effect of gold nanoparticle aggregation on cell uptake and toxicity." ACS Nano 5(7): 5478-5489.

Anand, A., Sharma, K. (2014). "Using current data to define new approach in age related macular degeneration: need to accelerate translational research." Curr Genomics 15(4): 266-277.

Andres-Guerrero, V., Zong, M., et al (2015). "Novel biodegradable polyesteramide microspheres for controlled drug delivery in Ophthalmology." J Control Release 211: 105-117.

Aubert-Pouessel, A., Bibby, D. C. (2002). "A novel in vitro delivery system for assessing the biological integrity of protein upon release from PLGA microspheres." Pharm Res 19(7): 1046-1051.

Avery, R. (2014). " Intravitreal Injection Technique And Monitoring Updated Guidelines of an Expert Panel." Retinal journal(34 sup.12).

Barcia, E., Herradon, C., Herrero-Vanrell, R (2005). "Biodegradable additives modulate ganciclovir release rate from PLGA microspheres destined to intraocular administration." Lett. Drug. Des. 2: 184-193.

Barcia, E., Herrero-Vanrell, R. (2009). "Downregulation of endotoxin-induced uveitis by intravitreal injection of polylactic-glycolic acid (PLGA) microspheres loaded with dexamethasone." Exp Eye Res 89(2): 238-245.

Behar-Cohen, F. (2013). "Drug Delivery to the Vitreous Humor". Ocular Drug Delivery, Systems Barriers and Application of Nanoparticulate Systems. Florida, CRC Press.

Bertram, J. P., Rauch, M. F. (2010). "Using polymer chemistry to modulate the delivery of neurotrophic factors from degradable microspheres: delivery of BDNF." Pharm Res 27(1): 82-91.

Bolitho, C., Bayl, P. (2007). "The anti-apoptotic activity of albumin for endothelium is mediated by a partially cryptic protein domain and reduced by inhibitors of G-coupled protein and PI-3 kinase, but is independent of radical scavenging or bound lipid." J Vasc Res 44(4): 313-324.

Checa-Casalengua, P., Jiang, C. (2011). "Retinal ganglion cells survival in a glaucoma model by GDNF/Vit E PLGA microspheres prepared according to a novel microencapsulation procedure." J Control Release 156(1): 92-100.

Cho, E. R., Kang, S. W. (2005). "Poly(lactic-co-glycolic acid) microspheres as a potential bulking agent for urological injection therapy: preliminary results." J Biomed Mater Res B Appl Biomater 72(1): 166-172.

Chovatiya, R., Medzhitov, R. (2014). "Stress, inflammation, and defense of homeostasis." Mol Cell 54(2): 281-288.

Coccoli, V., Luciani, A. (2008). "Engineering of poly(epsilon-caprolactone) microcarriers to modulate protein encapsulation capability and release kinetic." J Mater Sci Mater Med 19(4): 1703-1711.

Comyn, O., Lightman, S. L. (2013). "Corticosteroid intravitreal implants vs. ranibizumab for the treatment of vitreoretinal disease." Curr Opin Ophthalmol 24(3): 248-254.

Davis, M. E. (2009). "The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic." Mol Pharm 6(3): 659-668.

Dunne, M. M., Ramtoola, Z. (2009). "Fluphenazine release from biodegradable microparticles: characterization and modelling of release." J Microencapsul 26(5): 403-410.

Engin, K. N. (2009). "Alpha-tocopherol: looking beyond an antioxidant." Mol Vis 15: 855-860.

Fernandez-Carballido, A., Puebla, P. (2006). "Radiosterilisation of indomethacin PLGA/PEG-derivative microspheres: protective effects of low temperature during gamma-irradiation." Int J Pharm 313(1-2): 129-135.

Francis, G. L. (2010). "Albumin and mammalian cell culture: implications for biotechnology applications." Cytotechnology 62(1): 1-16.

Fratoddi, I. (2015). "The puzzle of toxicity of gold nanoparticles. The case-study of HeLa cells." Toxicol. Res. 4(4): 796-800.

Garbayo, E., Ansorena, E. (2008). "Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres." Eur J Pharm Biopharm 69(3): 844-851.

Garbayo, E., Montero-Menei, C. N. (2009). "Effective GDNF brain delivery using microspheres--a promising strategy for Parkinson's disease." J Control Release 135(2): 119-126.

Gaudana, R., et al (2010). "Ocular drug delivery." AAPS J 12(3): 348-360.

Gavini, E., Rassu, G. (2008). "Spray-dried microspheres based on methylpyrrolidinone chitosan as new carrier for nasal administration of metoclopramide." Eur J Pharm Biopharm 68(2): 245-252.

Giteau, A., Venier-Julienne, M. C. (2008). "How to achieve sustained and complete protein release from PLGA-based microparticles?" Int J Pharm 350(1-2): 14-26.

Gonciarz, A., Kus, K. (2012). "Capillary electrophoresis/frontal analysis versus equilibrium dialysis in dexamethasone sodium phosphate-serum albumin binding studies." Electrophoresis 33(22): 3323-3330.

Halliwell, B., Whiteman, M. (2004). "Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean?" British Journal of Pharmacology 142(2): 231-255.

He, J. T., et al (2006). "Stabilization and encapsulation of a staphylokinase variant (K35R) into poly(lactic-co-glycolic acid) microspheres." Int J Pharm 309: 101-108.

Herrero-Vanrell, R., Bravo-Osuna, I. (2014). "The potential of using biodegradable microspheres in retinal diseases and other intraocular pathologies." Prog Retin Eye Res 42: 27-43.

Huang, X., Li, N. (2015). "Quantitative three-dimensional analysis of poly (lactic-co-glycolic acid) microsphere using hard X-ray nano-tomography revealed correlation between structural parameters and drug burst release." J Pharm Biomed Anal 112: 43-49.

Jiang, C., Moore, M. J. (2007). "Intravitreal injections of GDNF-loaded biodegradable microspheres are neuroprotective in a rat model of glaucoma." Mol Vis 13: 1783-1792.

Klose, D., Siepmann, F. (2006). "How porosity and size affect the drug release mechanisms from PLGA-based microparticles." Int J Pharm 314(2): 198-206.

Lee, B. K., Yun, Y. H. (2012). "Fabrication of drug-loaded polymer microparticles with arbitrary geometries using a piezoelectric inkjet printing system." Int J Pharm 427(2): 305-310.

Madsen, C. G., Skov, A. (2015). "Simple measurements for prediction of drug release from polymer matrices - Solubility parameters and intrinsic viscosity." Eur J Pharm Biopharm 92: 1-7.

Martinez-Sancho, C., Herrero-Vanrell, R. (2003). "Poly (D,L-lactide-co-glycolide) microspheres for long-term intravitreal delivery of aciclovir: influence of fatty and non-fatty additives." J Microencapsul 20(6): 799-810.

Martinez-Sancho, C., Herrero-Vanrell, R. (2006). "Vitamin A palmitate and aciclovir biodegradable microspheres for intraocular sustained release." Int J Pharm 326(1-2): 100-106.

Miserocchi, E., Fogliato, G. (2013). "Review on the worldwide epidemiology of uveitis." Eur J Ophthalmol 23(5): 705-717.

Nita, M., Grzybowski, A. (2014). "Age-related macular degeneration in the aspect of chronic low-grade inflammation (pathophysiological parainflammation)." Mediators Inflamm 2014: 930671.

Rafi, M., Singh, S. M. (2010). "Controlled release of bioactive recombinant human growth hormone from PLGA microparticles." J Microencapsul 27(6): 552-560.

Rodrigues, L. B., Leite, H. F. (2009). "In vitro release and characterization of chitosan films as dexamethasone carrier." Int J Pharm 368(1-2): 1-6.

Rodríguez, J., Guzmán, M. (2013).Preliminary studies on HSA microparticles to modulate dexamethasone release. CRS Chapter Congress. Valencia

Sah, H. (1999). "Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation." J Control Release 58(2): 143-151.

Sampat, K. M., Garg, S. J. (2010). "Complications of intravitreal injections." Curr Opin Ophthalmol 21(3): 178-183.

Sapieha, P., Hamel, D. (2010). "Proliferative retinopathies: angiogenesis that blinds." Int J Biochem Cell Biol 42(1): 5-12.

Schiller, M., Franz, S. (2008). "Hypothesis: human serum-borne albumin bound lipids promote cellular survival after apoptosis induction by a variety of stimuli." Apoptosis 13(2): 319-328.

Schmidt, S., Gonzalez, D. (2010). "Significance of protein binding in pharmacokinetics and pharmacodynamics." J Pharm Sci 99(3): 1107-1122.

Shah, V. P., Tsong, Y. (1998). "In vitro dissolution profile comparison--statistics and analysis of the similarity factor, f2." Pharm Res 15(6): 889-896.

Sherif, Z., Pleyer, U. (2002). "Corticosteroids in ophthalmology: past-present-future." Ophthalmologica 216(5): 305-315.

Short, B. G. (2008). "Safety evaluation of ocular drug delivery formulations: techniques and practical considerations." Toxicol Pathol 36(1): 49-62.

Srivastava, S., Kumar, R. (2015). "Modified Release Biodegradable Polymeric Microspheres of Stavudine: Cell Viability, Cellular Uptake, Hemolysis Studies and In Vivo Pharmacokinetics." Curr HIV Res 13(6): 503-516.

van Wijngaarden, P., Qureshi, S. H. (2008). "Inhibitors of vascular endothelial growth factor (VEGF) in the management of neovascular age-related macular degeneration: a review of current practice." Clin Exp Optom 91(5): 427-437.

Wang, M. (2010). "A spheres-in-sphere structure for improving protein-loading poly (lactide-co-glycolide) microspheres." Polymer Degradation and Stability 95: 6-13.

Wang, Y., Gu, B. (2014). "Microspheres prepared with PLGA blends for delivery of dexamethasone for implantable medical devices." Pharm Res 31(2): 373-381.

Yao, J., Tucker, B. A. (2011). "Robust cell integration from co-transplantation of biodegradable MMP2-PLGA microspheres with retinal progenitor cells." Biomaterials 32(4): 1041-1050.

Yasukawa, T., Tabata, Y. (2011). "Recent advances in intraocular drug delivery systems." Recent Pat Drug Deliv Formul 5(1): 1-10.

Yeh, M. K., Chen, J. L. (2007). "The preparation of sustained release erythropoietin microparticle." J Microencapsul 24(1): 82-93.

Yuan, W., Wu, F. (2009). "Development of protein delivery microsphere system by a novel S/O/O/W multi-emulsion." Eur J Pharm Sci 36(2-3): 212-218.

Zhang, X., Zeng, H. (2014). "Diabetic macular edema: new concepts in patho-physiology and treatment." Cell Biosci 4: 27.

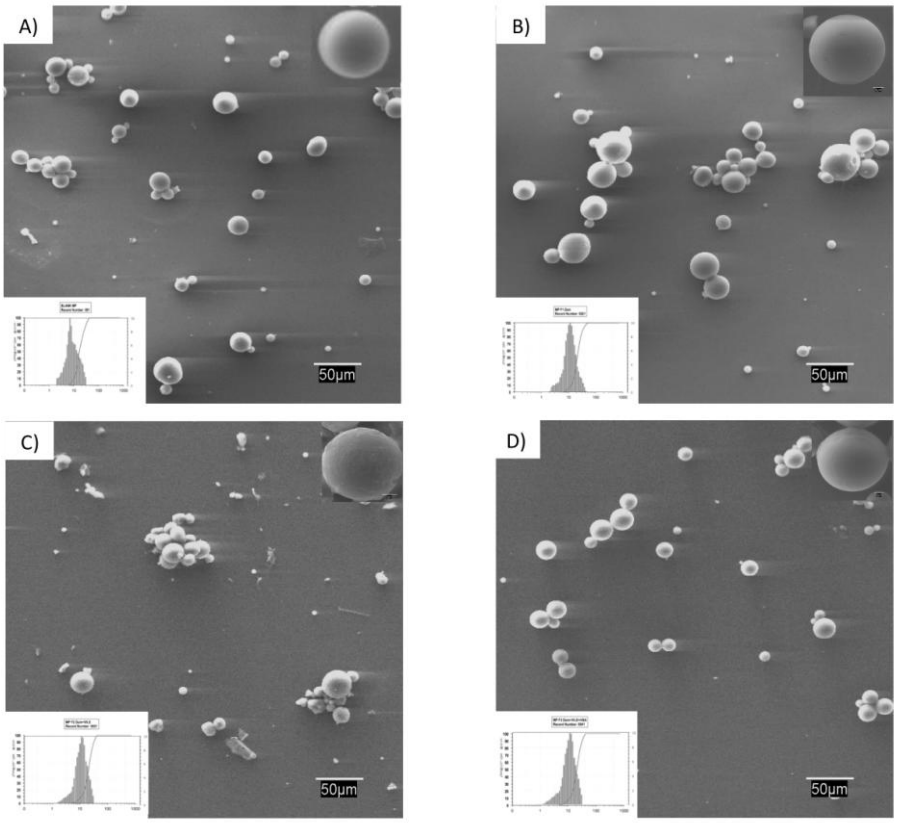


Figure 1

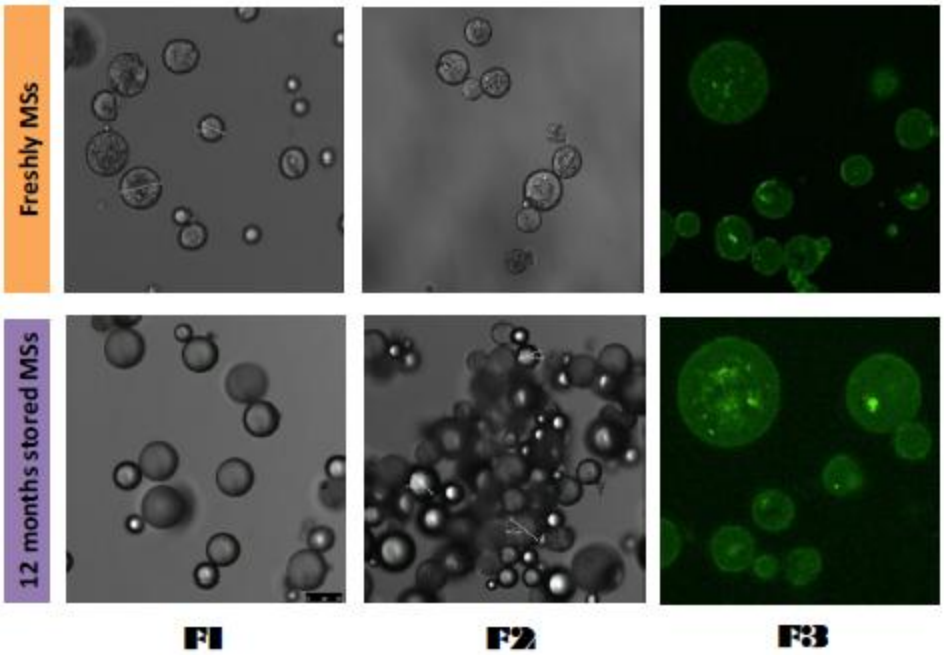


Figure 2

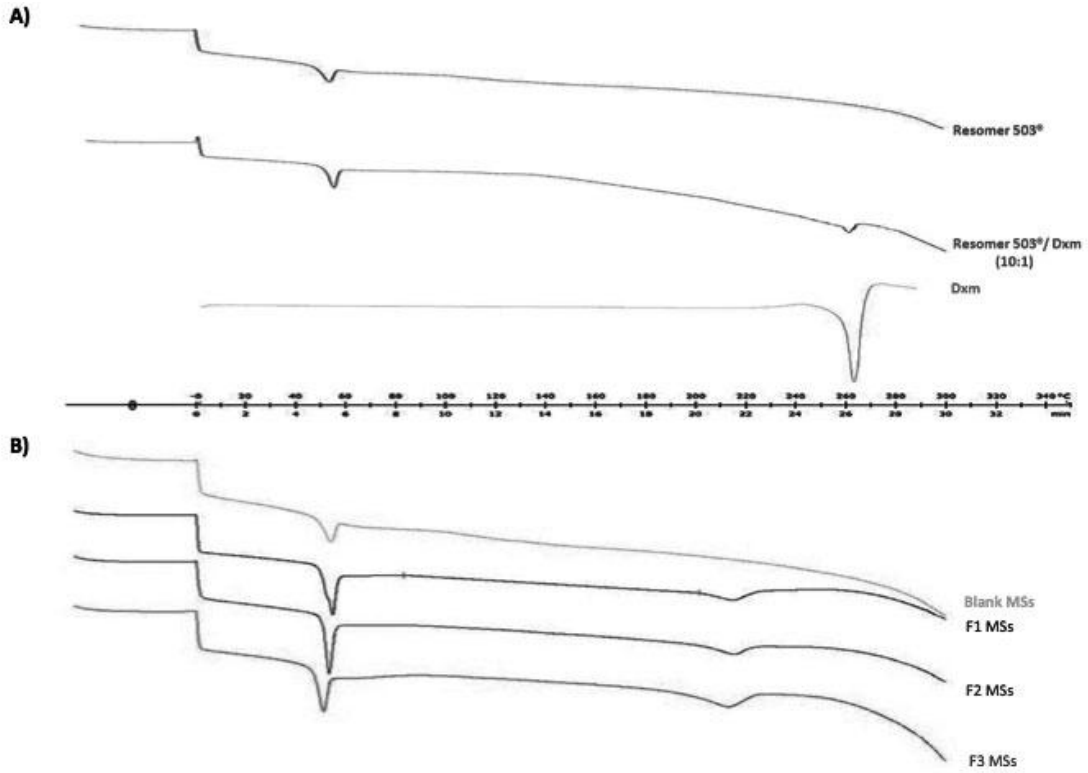


Figure 3

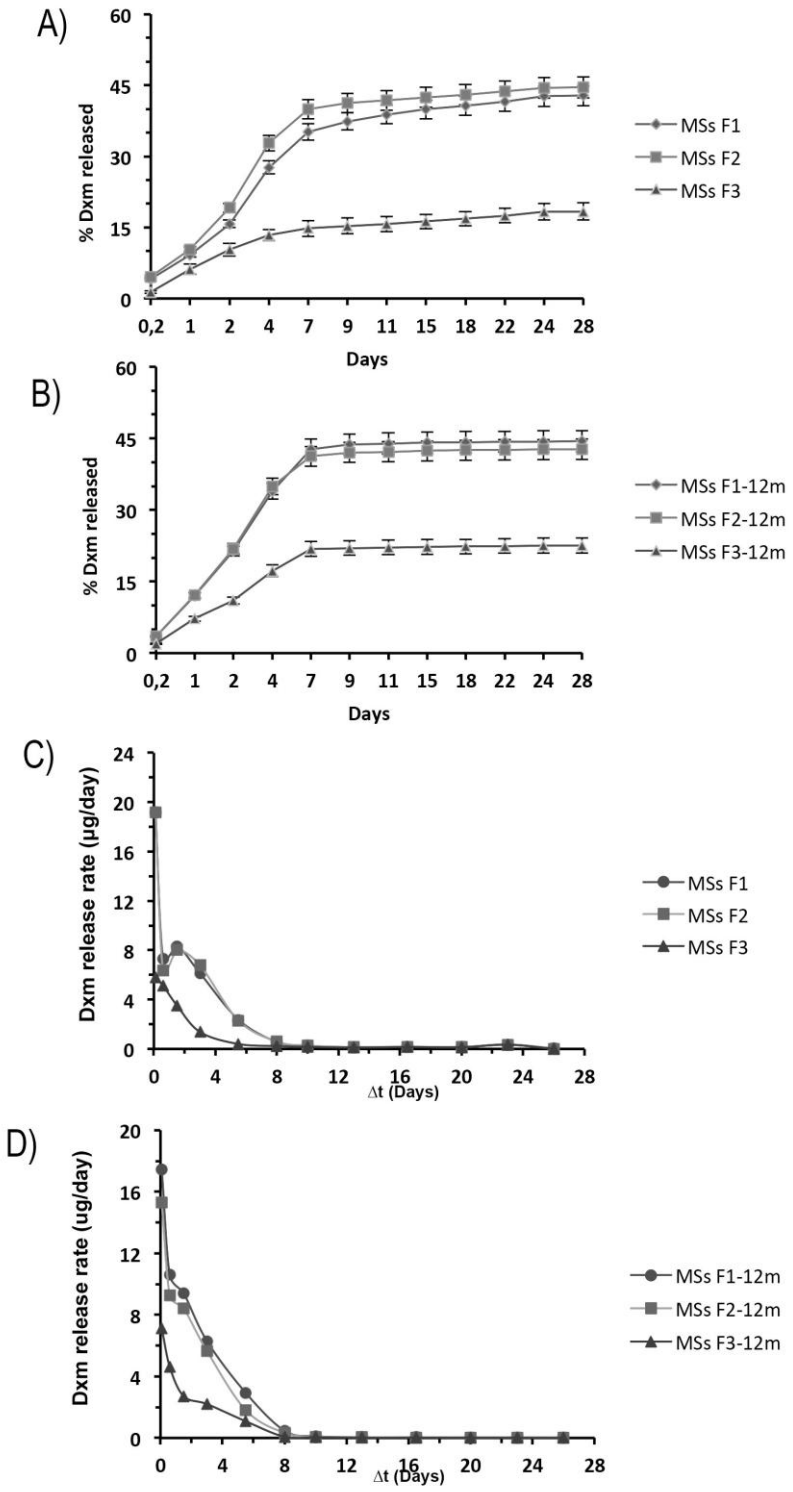


Figure 4

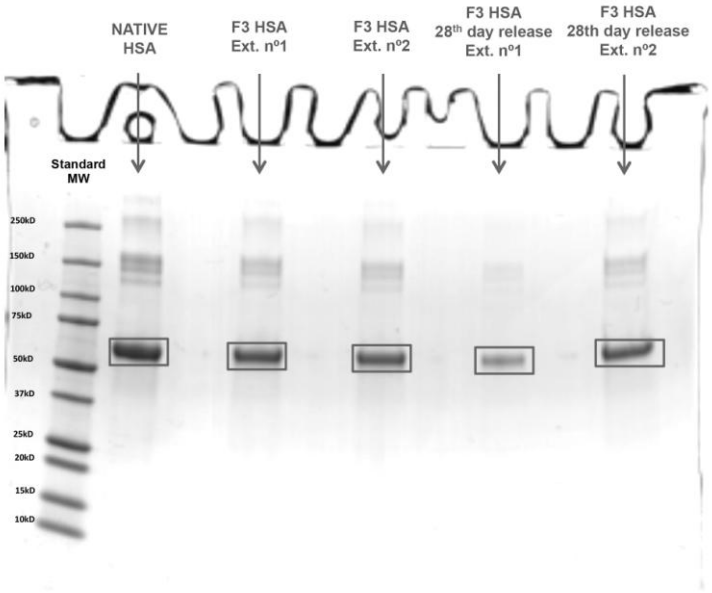


Figure 5

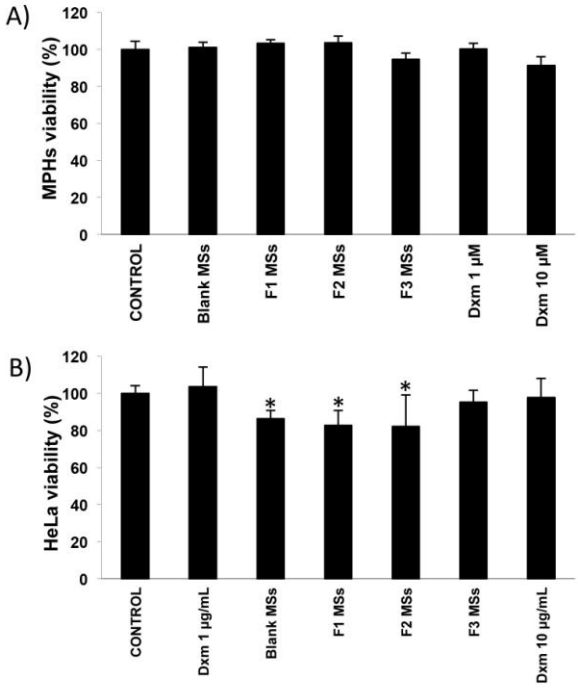


Figure 6

FIGURES

Fig. 1- SEM images of PLGA (50:50) MSs fraction sized 20-40μm: (A) Blank MSs, (B) F1, (C) F2 and (D) F3 Dxm-loaded MSs. Particle surface (top right) and particle size distribution (bottom left) are shown.

Fig. 2. Confocal laser microphotographs of F1, F2 and F3 MSs freshly prepared and 12 months later. Images from F3 MSs exhibit green fluorescence due to encapsulated FITC-HSA.

Fig. 3: Differential scanning calorimetry (DSC) at a heating rate of 10ºC/min for (A) raw materials PLGA (50:50), Dxm and PLGA /Dxm (10:1) physical mixture and B) Blank MSs, F1, F2 and F3 loaded MSs.

Fig.4 In vitro Dxm release profiles in PBS medium (37ºC, pH=7.4) versus time, represented as drug cumulative percentage and drug release rate: A) and C) are freshly prepared MSs and B) and D) are MSs after one year stored at 4ºC.

Fig.5 Gradient 4-20% Polyacrylamide Gel Electrophoresis (SDS-PAGE) image of HSA extracted from F3 MSs. The molecular markers (ladder) are in the left lane. Control line corresponds to native FITC-HSA, A) and B) lines show spots of two different extractive processes from freshly prepared F3 MSs; and C) and D) from F3 MSs on the 28th day of the release assay.

Fig.6 Effect of free Dxm solution (1 y 10 μM), blank MSs and F1, F2 and F3 Dxm-loaded MSs on culture cell viability (%) of A) Macrophage J-774.2 cell line (MPH) and B) HeLa cell line. *$p<0.05$

TABLES

Tab. 1. Influence of PLGA microsphere composition on mean particle size and distribution extent (percentiles and span). (Data are shown as $\overline{X} \pm SD$, n=6).

MSs formulation	PLGA (mg)	Dxm (mg)	Vit. E (mg)	FITC-HSA (mg)	Size (µm)	d_{10} ± S.D. (µm)	d_{50} ± S.D. (µm)	d_{95} ± S.D. (µm)	SPAN ± S.D.
BLANK	200	-	-	-	20.21 ± 7.83	14.39 ± 1.63	27.83 ± 1.26	45.37 ± 0.86	1.12 ± 0.08
F 1	200	20	-	-	18.82 ± 7.24	12.26 ± 1.20	28.50 ± 1.35	43.68 ± 0.56	1.11 ± 0.10
F 2	200	20	0,02	-	19.93 ± 8.50	14.31 ± 1.62	25.46 ± 1.35	47.27 ± 1.20	1.30 ± 0.09
F 3	200	20	0,02	0,02	22.37 ± 8.96	16.57 ± 1.14	29.06 ± 0.35	47.70 ± 0.65	1.07 ± 0.06

Tab. 2. Performance parameters of production and characterisation of PLGA (50:50) MSs (fraction 20-40 µm) freshly prepared (t=0m) and after storage for 12 m at 4ªC (t=12m). (Data are shown as $\overline{X} \pm SD$, n=6).

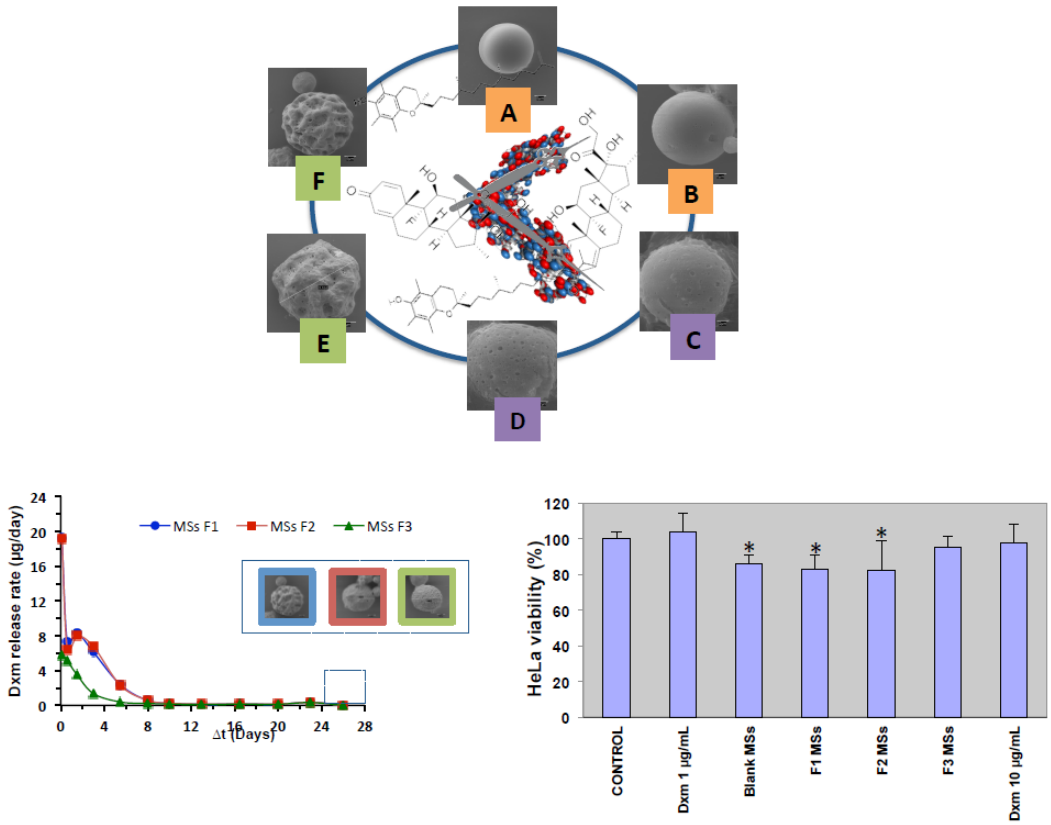
MSs formulation	Production yield (%)	Mean particle size (µm)	SPAN ± S.D.	Loading (µg/mgMS) Dxm [(a)] FITC-HSA [(b)]	Encapsulation Efficiency (%) Dxm [(a)] FITC-HSA [(b)]	ζ-Pot (mV) NaCl 0,9%
BLANK	45.9 ± 5.2	26.87 ± 0.14	0.68 ± 0.01	---------	----------	-9,4 ± 0,4
F1 (t = 0 m)	30.8 ± 4.4	27.22 ± 0.61	0.67 ± 0.02	90.5 ± 11.3[(a)]	99.5 ± 12.4[(a)]	-7,8 ± 0,2
F 2 (t = 0 m)	30.1 ± 3.7	27.72 ± 0.55	0.67 ± 0.01	80.9 ± 14.1[(a)]	89.0 ± 15.5[(a)]	-10,4 ± 1,0
F 3 (t = 0 m)	41.4 ± 4.0	26.82 ± 0.22	0.70 ± 0.01	84.8 ± 12.7[(a)] 0.113 ± 0.001[(b)]	93.3 ± 13.9[(a)] 71.45 ± 2.01[(b)]	-11,9 ± 0,8
F1 (t = 12 m)	-	28.01 ± 0.82	0.67 ± 0.02	89.8 ± 12.3[(a)]	99.2 ± 12.7[(a)]	-7,9 ± 0,2
F2 (t = 12 m)	-	28.10 ± 0.78	0.68 ± 0.02	81.0 ± 15.5[(a)]	89.5 ± 15.9[(a)]	-9,1 ± 0,2
F3 (t = 12 m)	-	27.07 ± 0.31	0.71 ± 0.02	85.2 ± 13.5[(a)] 0.110 ± 0.001[(b)]	93.6 ± 14.1[(a)] 67.21 ± 2.77[(b)]	-10,4 ± 1,0

Tab. 3. DSC thermal events for raw solid materials and PLGA (50:50) MSs formulations ***(∆Hm were normalized to weight)***.

	Tg (ºC)	***∆Hm$_{(PLGA)}$ (Jg^{-1})***	***Tm (ºC)***	***∆Hm$_{(Dxm)}$ (Jg^{-1})***
PLGA (50:50)	53.8	4.10	------	------
Dxm	------	------	262.5	149.4
PLGA- Dxm (10:1)	54.3	3.91	260.4	2.8
Blank MSs	53.8	4.00	------	------
MSs F1	54.4	7.50	214.7	3.5
MSs F2	53.2	6.79	215.0	3.4
MSs F3	51.1	6.19	212.8	5.4

Tab. 4. In vitro drug release studies for PLGA MSs (20-40 μm) freshly prepared and after 12 months storage at 4ºC. Data shown are: Dxm burst effect at first 5h (%), percentage released (%) and content retained (μg/mg MS) after incubation for 28 days at 37ºC in PBS medium for Dxm [(a)] and FITC-HSA[(b)] and Dxm release rate constants, K_1 and K_2, obtained from data fitting to proposed biexponential release model. (Data are shown as $\bar{X} \pm SD$, n=6).

MSs formulation	Dxm Burst Effect (%)	Released at 28d (%)	Retained at 28d (μg/mgMS)	K_1 (d^{-1})	K_2 (d^{-1})	R^2
F1 (t =0 m)	3.50 ± 0.79	42.80 ± 2.96 [(a)]	58.66 ± 2.24 [(a)]	0.701 ± 0.118	0.173 ± 0.025	0.9864
F2 (t =0 m)	4.60 ± 1.64	44.56 ± 1.44 [(a)]	43.74 ± 2.41[(a)]	0.782 ± 0.164	0.183 ± 0.021	0.9862
F3 (t=0 m)	1.36 ± 0.26	18.39 ± 1.82 [(a)] 37.46 ± 1.79 [(b)]	63.75 ± 1.40 [(a)] 0.071 ± 0.002 [(b)]	0.554 ± 0.118	0.280 ± 0.025	0.9707
F1 (t =12 m)	3.51 ± 0.25	44.86 ± 2.78 [(a)]	58.04 ± 2.46 [(a)]	0.789 ± 0.231	0.262 ± 0.040	0.9890
F2 (t =12 m)	3.78 ± 0.53	42.72 ± 2.16 [(a)]	46.81 ± 1.91 [(a)]	0.733 ± 0.350	0.216 ± 0.061	0.9801
F3 (t=12 m)	1.67 ±0.12	22.54 ± 2.07 [(a)] 38.26 ± 1.92 [(b)]	53.48 ± 2.05 [(a)] 0.068 ± 0.001 [(b)]	0.618 ± 0.059	0.221 ± 0.035	0.9760



Graphical abstract