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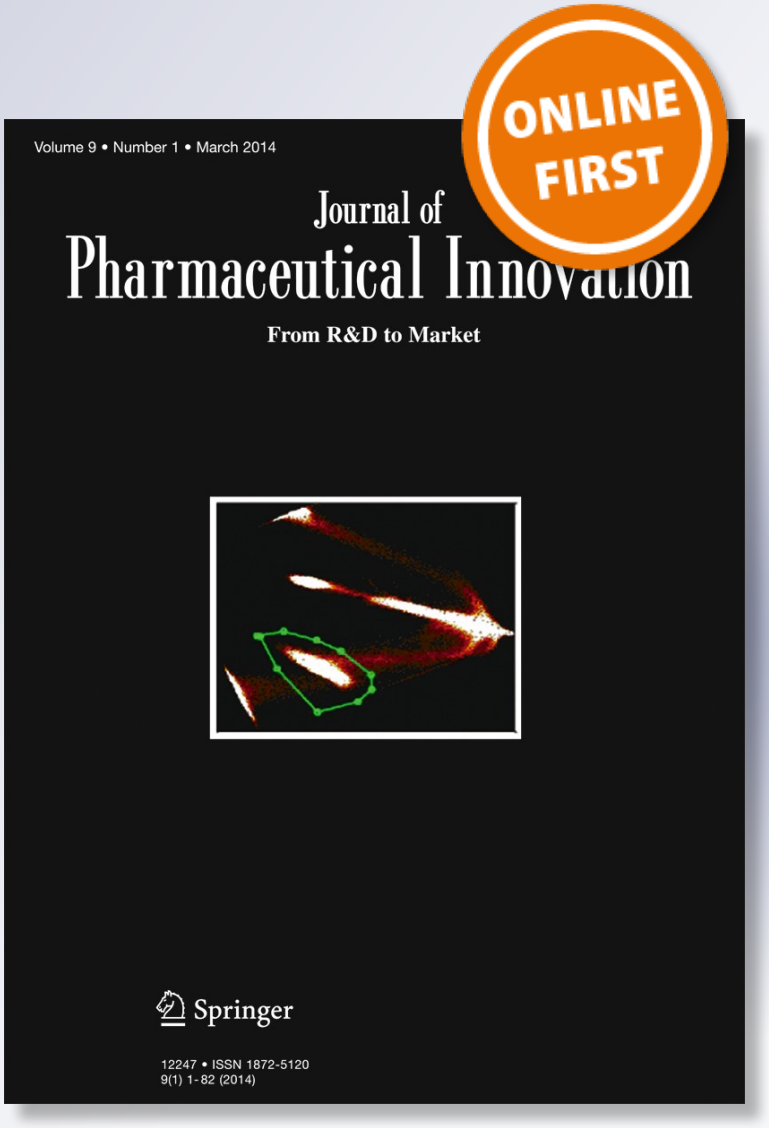

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RESEARCH ARTICLE

In Situ-Formed Microparticles of PLGA from O/W Emulsions Stabilized with PVA: Encapsulation and Controlled Release of Progesterone

L. N. Turino · R. N. Mariano · S. Boimvaser · J. A. Luna

Abstract In situ-formed microspheres are an alternative to expensive and complex manufactured preformed systems for the controlled release of drugs. The aim of this study was to evaluate the potential of stable O/W emulsions to entrap progesterone after in vitro precipitation of poly(D,L-lactide-co-glycolide) (PLGA) microparticles. This was achieved by a solvent selection based on their miscibility and capability to solubilize the drug and PLGA. Stability assays, size distribution studies, and progesterone encapsulation efficiency evaluation were carried out for the candidate formulations. After selection of the most suitable formulations, in vitro-controlled release test of progesterone were done. Results demonstrate that emulsions based on triacetin and polyvinyl alcohol (PVA) aqueous solutions were useful solvent systems to obtain microspheres capable to deliver the hormone in a controlled release manner. In addition, for the first time, for these authors, PVA was successfully implemented into a continuous phase to increase the stability of in situ-formed O/W formulations.

Keywords In situ-forming microspheres · Emulsion stability · Progesterone · PLGA · PVA

Introduction

Progesterone is a lipophilic steroid hormone with a low molecular weight (314 Da). Long-time controlled delivery systems of this drug had been proposed for regulating the estrous cycle of women, cows, and sheep by incorporating progesterone into biodegradable and non-biodegradable polymeric matrices [1-4]. Poly(D,L-lactide-co-glycolide) (PLGA) is a hydrophobic, biodegradable, and biocompatible (FDA approved) polymer. Its degradation kinetic and consequently, the rate and time of drug release from the polymeric matrix can be manipulated by varying co-polymer composition, molecular weight, and end group of polymeric chains [5-8]. These special characteristics make PLGA a proper polymer to progesterone entrapment and delivery.

Preformed biodegradable microspheres for the controlled release of drugs are highly developed systems but with some disadvantages. Elaboration processes such as simple and double emulsion extraction/evaporation method, coacervation, spray drying, fluidization, etc., and the final lyophilization are expensive. Toxic and environmental dangerous organic solvents are used to dissolve the polymer, and residues of these solvents in the final product are difficult to remove. In addition, microspheres have to be suspended in an aqueous or oily medium before administration. In situ-formed systems have been developed as an alternative to preformed microspheres. The mechanism of in situ-forming technology is based on the immiscibility of some polymers in water or physiological media. A solution of these polymers that makes contact with an aqueous medium precipitates by solvent exchange, i.e., the solvent of the polymeric solution diffuses into the surrounding aqueous fluid while water diffuses into the polymer [9, 10]. Shape of precipitated product depends on formulation characteristics and in vivo conditions at the site of injection [11, 12]. A fluid emulsion should be parenterally administered to obtain microspheres [13, 14]. The emulsion should consist of two phases: an organic (O) phase and other which could be oily (OIL) or aqueous (W). The first is called dispersed phase or phase 1 and the second continuous phase or phase 2. Thus, O/OIL and O/W emulsions can be developed with some particular requirements: (i) solvents of both phases should be biocompatible; (ii) polymer and drug should be

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soluble only in phase 1; (iii) phase 1 also could dissolve surfactants; and (iv) phase 2 should only dissolve surfactants. These characteristics would reduce precipitation of agglomerate/implant, promote microspheres formation, and entrap high quantities of drug. Furthermore, to increase the chances of entrapping a hydrophilic drug in a microsphere, the emulsions should be O/OIL. This is possible because the drug is surrounded by an oily barrier that isolates it from aqueous external environment and restricts drug precipitation within the microsphere limits [13-16]. For lipophilic/hydrophobic drugs, O/W emulsions have demonstrated to be efficient for encapsulating more than 50 % of the initial drug depending on studied PLGA concentration [17, 18]. Some studies reported the efficiency of systems based on in situ-formed PLGA matrices for the entrapment of proteins [14, 19], peptides [20, 21], anesthetic agents [22], antimicrobial agents [23], antipsychotic [24], and anticancer drugs [11]. Other authors studied the in situ precipitation of poly(ethylene glycol) or poly(propylene glycol) diacrylate [25] and PLGA [26] implants for the controlled release of progesterone. To our knowledge, progesterone encapsulation within in situ-formed microspheres of PLGA has not been reported in the literature.

The main disadvantage during emulsion development is ensuring its stability. Reversible or irreversible phenomena that commonly affect the stability of emulsions include: (i) cremation, the dispersed phase is concentrated at the top of the emulsion; (ii) sedimentation, the dispersed phase is concentrated at the bottom of the emulsion; (iii) flocculation, the microglobules (dispersed phase) form aggregates that do not fuse together; and (iv) coalescence, the microglobules are fused together. Coalescence involves the complete separation of emulsion phases and is irreversible. In a simplified way, the mechanisms of emulsion breaking are studied as a single droplet of dispersed phase that approaches to a coalesced continuous phase. As cremation or sedimentation is the first step of this mechanism, retarding this process is equivalent to increase stability, or at least make coalescence slower. In this way, the well-known Stokes' law describes the rate of the upward or downward motion of a droplet as a function of droplet radius. Under steady-state conditions, the so-called Stokes' velocity explains the phenomenon of cremation or sedimentation as a function of the density and viscosity of the emulsion phases [27]:

$$\nu = \frac{d^2(\rho - \rho_0)g}{18\eta_0} \quad (1)$$

in which ν is the rate of sedimentation or cremation, d is the diameter of the dispersed phase, ρ and ρ_0 are the densities of phases 1 and 2, respectively, η_0 is the viscosity of phase 2, and g is the acceleration due to gravity. In this regard, it is usual the addition of surfactants or stabilizers into one or both phases to prevent the coalescence of microglobules, maintain its spherical shape, and facilitate the removal of emulsion's solvents into the aqueous surrounding medium during the In situ precipitation of PLGA microparticles [13, 24, 28]. Polyvinyl alcohol (PVA) is the most commonly used emulsifier in drug encapsulation techniques based on simple and double emulsion extraction/evaporation methods for preformed microsphere synthesis [18, 29]. As far as the authors are aware, its incorporation for stabilizing in situ-formed microparticles of PLGA has not been reported in the literature.

Based on the hydrophobic nature of progesterone, in the present contribution, we focus on the development of stable O/W emulsions suitable with in situ-formed systems. In this regard, we evaluate the utility of some organic solvents and PVA aqueous solutions to conform phases 1 and 2 of stable emulsions, respectively. The shape and size of in situ-formed microparticles of PLGA, entrapment efficiency, and controlled release of progesterone were also studied.

Materials and Methods

Materials

The following chemicals were used: poly(D,L-lactide-co-glycolide) (PLGA; uncapped low molecular weight 50:50 PLGA; RESOMER RG 502H, Boehringer Ingelheim Pharma KG, Germany); progesterone (PRG; 99.0 %, Farmabase Pharmaceutical Raw Materials, Italy); N-methyl-2-pyrrolidone, dimethyl sulfoxide, glycerol formal, 2-pyrrolidone, triacetin, and polyethylene glycol 400 (Sigma-Aldrich, Argentina); benzyl alcohol and methylene chloride (CICCARELLI, Argentina); Poloxamer 188 (P188; Rumapel, Argentina); and polyvinyl alcohol (PVA; 205 kDa; hydrolysis of 88.3 %; SERQUIM, Argentina). The water used in emulsions, solutions, and dilutions, was ultrapure.

Miscibility

Equal volumes of studied phases (organic solvent and water) were placed in graduated glass vials to evaluate the O/W miscibility. Vials were manually shaken and kept at 25 °C for 24 h. The total or partial miscibility was evaluated: miscible (+)=one homogeneous phase; partially miscible (+/−)= two phases, one bigger than other; immiscible (−)=two equal phases. Solvent evaporation was not observed during assay.

Solubility of PLGA

The method described by Ferruti et al. [30] was implemented with modifications. In sealed glass vials, 100 mg of PLGA and 1 mL of studied solvent were magnetic stirred and kept at

50 °C for 1 h. Solubility of PLGA was determined: soluble (S)=one homogeneous phase; partially soluble (PS)=remaining undissolved polymer; and insoluble (I)=undissolved polymer. Only methylene chloride was evaluated at 25 °C because of its low boiling point.

Solubility of Progesterone

Solubility of progesterone was studied at 25 °C taking into account the method proposed by Kranz and Bodmeier [31] with modifications. An excess drug was put in contact with 8 mL of the studied solvent and was homogenized by ultrasound for 1 h. After 3, 7, and 24 h, supernatant samples were withdrawn to corroborate that the system reached the equilibrium. Assays were conducted by quadruplicate. Progesterone concentration was analyzed by HPLC (Shimadzu LC-10A equipment) with UV detection by diode array. A Spherisorb C18 ODS2 column (4.6×250 mm; 5 μm) was used with ethanol/water (75:25) as the mobile phase at a flow rate of 0.9 mL min^{-1}. Oven temperature and detection wavelength of the assay were 35 °C and 254 nm, respectively. The evaluation of performance of the method showed that the model can explain 99.7 % (R^2) of the variation in the response variable. Also, calibration curve had an intra-assay coefficient of variation (CV) of <3.5 %, an inter-assay CV of <5.2 %, and a sensitivity equal to 0.1 ppm.

Density and Viscosity

Viscosity and density of phases 1 and 2 candidates were evaluated. Viscosity measurements were performed at 25 °C using a Cannon LV-2020 viscometer with a coupled Cannon CIC UL P171721 spindle. Density was evaluated at 25 °C with a 5-mL calibrated pycnometer (FITE, Argentina).

Preparation of Emulsions and In Situ-Formed Microparticles

Different emulsions were obtained following these steps: (i) After PLGA solubilization in phase 1, progesterone was added and the solution was magnetic stirred until complete drug dissolution; (ii) the solvent/PLGA/progesterone system was dropped into phase 2 and stirred with an Ultra-Turrax T25D homogenizer (dispersing element S25N-18G, IKA, Germany) for 2 min at 5,000 rpm. All the emulsions were obtained at room temperature with a phase 1/phase 2 ratio of 1:1, *v/v*. Emulsions were numbered in order of appearance with a formulation number (FNº).

For in vitro microparticles precipitation, 2 mL of emulsion were dropped into 50 mL of 0.1-M sodium phosphate buffer pH 7.4. Then, this mixture was kept at 37 °C and shaken at 100 rpm for 2 h. Microspheres were collected by centrifugation at 2,000 rpm for 5 min and preserved at 4 °C until further analysis.

Emulsions and Microparticles Characterization

Stability

Emulsions were placed in a graduated glass collector of 25 mL and observed every 5 min during 2 h. If a phase separation bigger than 10 % of total volume appeared, the time was registered as a measure of stability.

Shape and Size Distribution: Optical Microscopy

Samples of microparticles suspension were observed in a Leica DM2500 M microscope with a coupled camera Leica DFC 290 HD. Diameters of approximately 100 microspheres were measured in the photomicrographs using an image processing program (ImageJ 1.40 g, National Institutes of Health).

Morphology: Scanning Electron Microscopy

Microparticles were resuspended in water and one drop of this suspension was put over an aluminum stub until water evaporation. Samples were then sputter coated with gold under argon atmosphere (SPI Supplies, 12157-AX), using soft conditions (two sputterings of 40 s each, with an intensity of 15 mA). The morphology of microparticles was examined using an acceleration voltage of 20 kV, in a JEOL JSM-35C equipped with the image acquisition program JEOL SemAfore.

In Vitro Encapsulation Efficiency

Approximately 100 mg of microparticles containing progesterone were washed with 5 mL of hexane/isopropyl alcohol (1:1) as Jain et al. [14] reported. Then, they were dried under vacuum, weighed (*Mm*) and dissolved in an appropriate volume of methylene chloride. Mass of progesterone (*Mp*) in methylene chloride solution was evaluated at 239 nm with a UV-VIS Shimadzu 2401 PC spectrophotometer. Calibration curve had an R^2 of 0.995, an intra-assay CV of <0.25 %, an inter-assay CV of <13.5 %, and a sensitivity equal to 0.1 ppm.

The quantity of entrapped drug (*En*) was calculated based on Eq. 2.

$$\%En = \frac{Mp}{(Mm - Mp)} 100 \tag{2}$$

in which *En* is grams of entrapped progesterone in 100 g of PLGA (% w w^{-1}).

For each formulation, results from Eq. 2 were compared with its initial load of progesterone (*Eni;* grams of initial

Table 1 Solubility of PLGA, solubility of progesterone, and miscibility in water of candidate solvents to conform O/W emulsions

Solvent	Solubility of PLGA (50 °C)	Solubility of PRG*(25 °C) (g L^{-1})	Miscibility in water
2-Pyrrolidone	S	85.3 (7.3)	+
Benzyl alcohol	S	423.2[b]	−
Methylene chloride	S[a]	ND	−
Dimethyl sulfoxide	PS	50.7 (5.2)	+
Glycerol formal	S	120.5 (7.0)	+
N-methyl-2-pyrrolidone	S	171.4 (12.3)	+
Polyethylene glycol 400	PS	14.8 (0.5)	+
Triacetin	S	28.4 (1.0)	+/−
Water	I	0.0117 (4×10^{-4})	+

ND not determined

*Mean (SD); n=4

[a] Assayed at 25 °C

[b] Data from Hammad and Müller [42]

progesterone in 100 g of PLGA; % w w^{-1}) to determine encapsulation efficiency (*Ee*) (see Eq. 3).

$$\%Ee = \frac{En}{Eni}\,100 \tag{3}$$

The study was carried out by triplicate.

In Vitro Drug Release

A known volume of emulsion (equivalent to a dose of 20 mg of progesterone) was added to glass flasks containing 75 mL of 0.1-M sodium phosphate buffer pH 7.4. Tween80 (1 %) and sodium azide (0.02 %) were added to increase progesterone solubility and to prevent decomposition of release medium, respectively. The sealed flasks were incubated at 37 °C under orbital shaking at 50 rpm. Sampling was performed after centrifugation of the release medium (2,500 rpm for 8 min) at 6, 12, and 24 h, and then once a day until day 5. At each time-point, an aliquot sample (0.5 mL) was taken and 50 % of the release medium was replaced by fresh medium to maintain sink conditions. Hormone concentration at each time-point was quantified by HPLC with the methodology detailed in the "Solubility of Progesterone" section.

Statistical Analysis

Shapiro–Wilk and chi-square test were used to determine if the data are well modeled by a normal distribution. Results were reported as follows: mean±standard deviation, for data exhibiting normal distributions; and median and interquartile range (IR, quartile 1–3), for data that did not follow a normal distribution. Mann–Whitney (Wilcoxon) and Kruskal–Wallis tests were implemented to median comparison between two or more assays, respectively. ANOVA and Fisher (LSD) tests were used to compare means between different assays. Goodness of fit was evaluated through the standard correlation coefficient (R^2). Error bars of the experimental data points in the graphics represent the standard deviations.

Results and Discussion

The potential use of different solvents to prepare O/W emulsions was evaluated. Their capability to dissolve PLGA and progesterone and their miscibility in water were studied (Table 1). All the analyzed solvents showed good capability for dissolve the polymer, except dimethyl sulfoxide and polyethylene glycol. Drug solubility in water was approximately 12 ppm. Thus, all solvents from Table 1 dissolve more drug than aqueous phase2, preventing a flow

Table 2 Stability of O/W (1:1) systems and size of in situ-formed microparticles

FNº	Emulsion				Microparticles
	Phase 1	Phase 2	PLGA (% w v^{-1})	Stability (min)	Size* (μm)
1	Benzyl alcohol	Water	10	25	Did not precipitate
2	Benzyl alcohol		20	15	Did not precipitate
3	Triacetin		10	5	Agglomerates/implants
4	Triacetin		20	5	Agglomerates/implants
5	Triacetin	0.4 % P188	20	20	46.8 (34.7)
6	Triacetin	2 % PVA	20	>2,880	<10

*Median (IR)

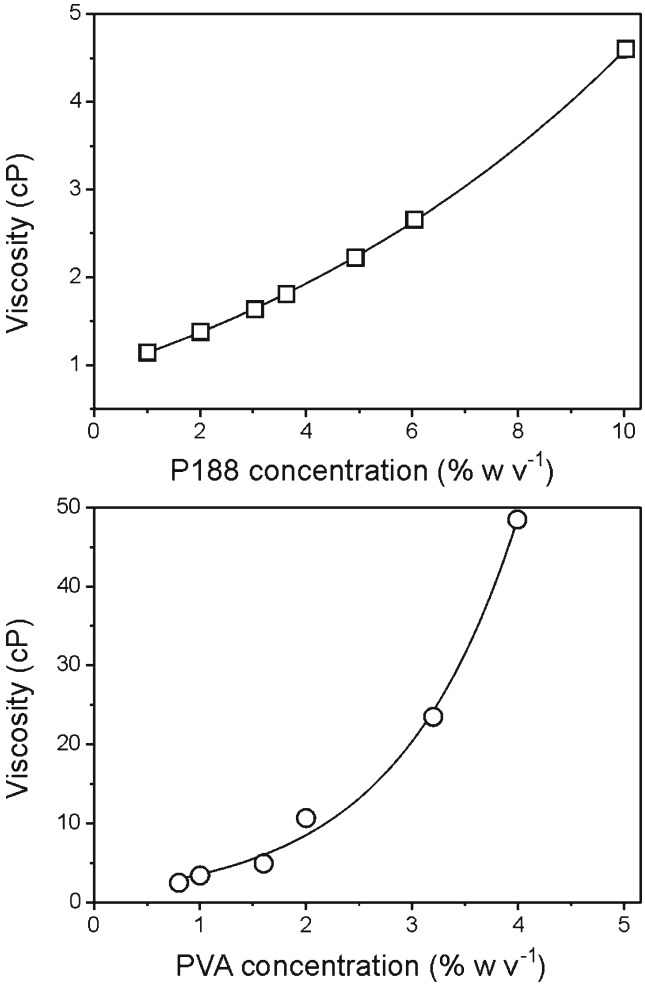

Fig. 1 Viscosity at 25 °C as a function of concentration of (*squares*) P188 and (*circles*) PVA in aqueous solutions. *Filled line* represents exponential fit for each distribution of experimental points

of hormone from dispersed to continuous phase during emulsification. As polymeric phase have to be immiscible in aqueous phase 2, the number of candidate solvents was reduced when miscibility in water was analyzed. According to Table 1, O/W emulsions could be obtained only using benzyl alcohol, methylene chloride, or triacetin. Despite the fact that PLGA was soluble in methylene chloride at room temperature, it was not used as phase 1 solvent because its low vapor pressure put in risk the robustness of the formulation. In addition, the International Conference on Harmonization (European Medicines Agency [EMA]) [32] classifies methylene chloride as class 2 solvent and its use has to be limited to protect patients against potential risks. On the other hand, triacetin is not toxic for animals after oral, topical, and parenteral exposure and is quickly metabolized into glycerol and acetic acid [33], and benzyl alcohol was approved for veterinary parenteral application by EMA.

Table 2 shows the stability of emulsions based on benzyl alcohol and triacetin without progesterone and size of its related microparticles. Formulations composed of benzyl alcohol at two concentrations of PLGA (FNº 1–2) did not precipitate after 120 min (time set for hardening of dispersed phase; see "Preparation of Emulsions and In Situ-Formed Microparticles" section). This could be explained by the low solubility of benzyl alcohol in water (see Table 1). When triacetin is analyzed, the lack of stability when water was used as phase 2 resulted in the precipitation of no particulate material, even when different concentrations of PLGA were used (FNº 3–4). We evaluated, therefore, the addition of additives into phase 2 to stabilize the system. Both stability and microparticles precipitation were improved when 0.4 % of P188 was added to FNº 5, but 20 min was still considered short for these authors.

Stokes' law (Eq. 1) indicates that stability could be improved ($< \nu$) if phases with equal densities are used, viscosity of phase 2 is incremented, or droplet size of dispersed phase is reduced. Also, Eq. 1 indicates that the difference between ρ and ρ_0 will determine if cremation or sedimentation occurs. In this way, densities of the candidate phase 1 (20 % PLGA in triacetin) and phase 2 (aqueous solutions of P188 and PVA) were evaluated. In a concentration range between 0.5 to 10 %w v^{-1}, densities of P188 and PVA solutions did not exceed 1.02 g mL^{-1}, which were lower than the density of phase 1=1.19 g mL^{-1}. This result reveals the prevalence of sedimentation phenomenon. To replace triacetin, there were no solvent in Table 1 that allows both obtaining a less dense phase1 and satisfying emulsion's requirements. For this reason, to counteract sedimentation the increase of phase 2

Table 3 Size, entrapment (*En*), and encapsulation efficiency (*Ee*) of in situ-formed microparticles from O/W (1:1) emulsions

FNº	Emulsion				Microparticles		
	Phase1	Phase2	PLGA (% w v^{-1})	*Eni* (% w w^{-1})	Size* (μm)	*En* ** (% w w^{-1})	*Ee* ** (%)
7	Triacetin	1 % PVA	10	25	12.9*** (7.3)	9.4 (1.5)	38.4 (6.1)
8			20	5	26.3*** (17.5)	1.9 (0.1)	37.8 (0.4)
9	Triacetin	2 % PVA	10	25	<5	7.7 (0.4)	30.9 (1.5)
10			20	5	6.5*** (6.0)	1.5 (0.3)	29.1 (6.3)

*Median (IR)

**Mean (SD); $n=3$

***Median statistically different ($P<0.05$)

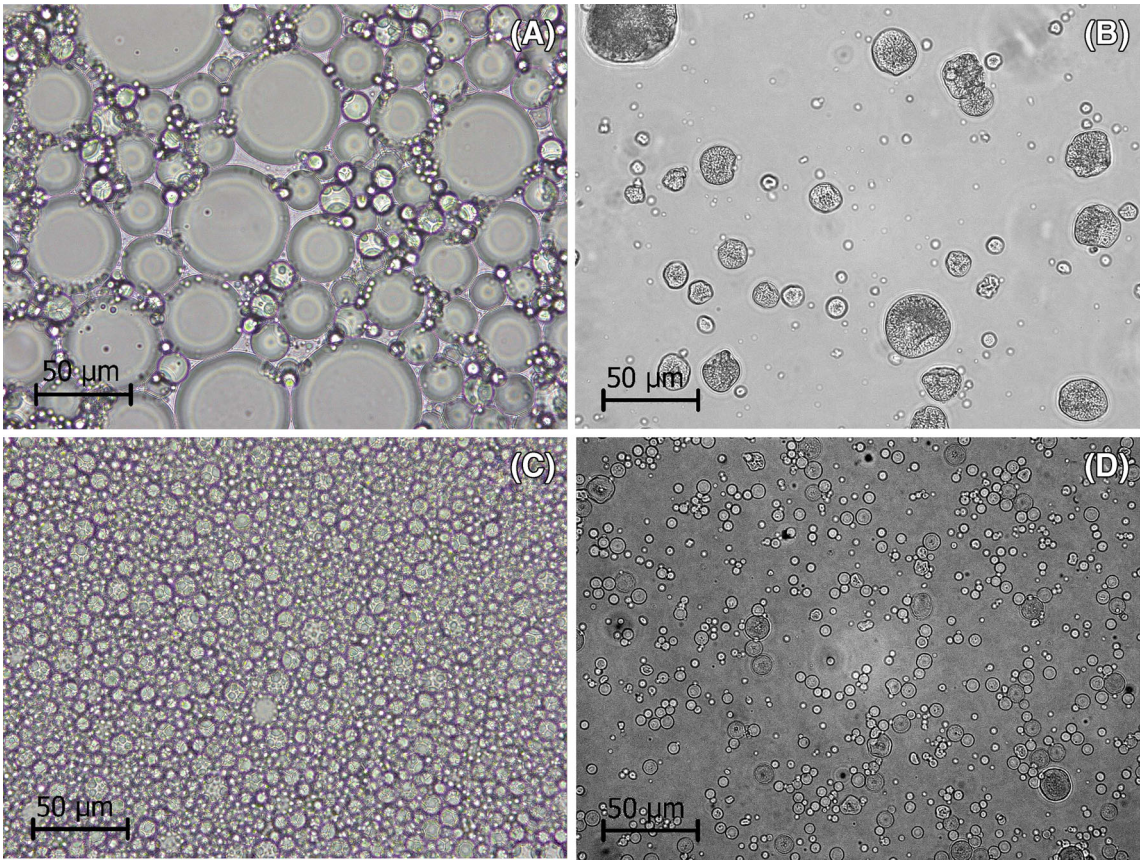

Fig. 2 Optic microphotography of (**a**, **c**) emulsions and (**b**, **d**) microspheres obtained from TR/PVA systems with 20 % of PLGA and 5 % of PRG. (**a**, **b**) 1 % PVA, FNº 8; (**c**, **d**) 2 % PVA, FNº 10

viscosity and/or the decrease of microglobules size of dispersed phase were studied. Figure 1 shows viscosity of aqueous solutions containing P188 and PVA. Exponential growth of viscosity was observed when additive concentrations were increased (R^2=0.9965 and 0.9846 for P188 and PVA, respectively), being higher for PVA than for P188. In fact, an emulsion based on 20 % PLGA in triacetin and 2 % PVA was stable for more than 48 h and microparticles median size was <10 μm after in vitro precipitation (see FNº 6 in Table 2). At this concentration of additive in phase 2, PVA resulted in an aqueous solution almost eight times more viscous than P188 (see Fig. 1), in accordance with an increase in stability (see Eq. 1).

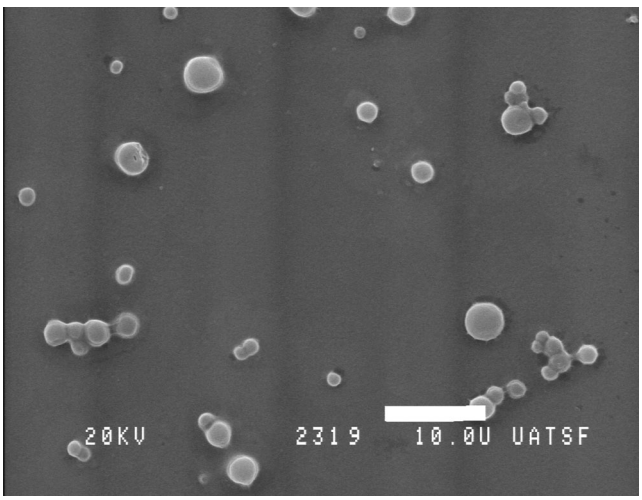

Fig. 3 Scanning electron microscopy of microparticles from FNº 10 prepared by solvent exchange. *Scale bar*=10 μm

Table 3 shows size distribution, *En* and *Ee* of progesterone for in situ-formed microparticles from O/W (1:1) emulsions based on 1 and 2 % of PVA (phase 2). Microparticles from all formulations resulted spherical and not aggregated. Nevertheless, all the formulations were observed by optical microscopy; Fig. 2 shows microphotography of emulsion and microparticles from FNº 8 and 10. For FNº 9, microspheres were so small that it was not possible to evaluate its size distribution without losing precision with the methodology applied. The size of the remaining particles (FNº 7, 8, and 10) did not show a normal size distribution (P<0.05). The increment of PVA concentration in aqueous phase caused a decrease in the median size of microparticles at the two studied concentrations of PLGA, as previously reported for other microsphere synthesis methods [34, 35]. For preformed microspheres, it has been reported that PVA is adsorbed on the polymeric particle surface during precipitation, forming a layer that would stabilize the

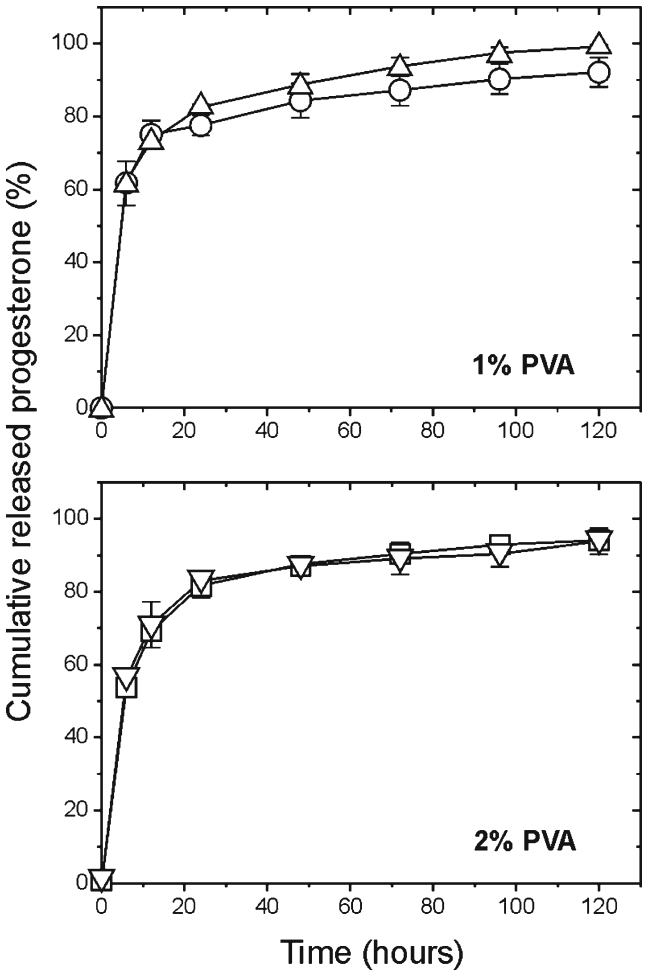

Fig. 4 Progesterone release from in situ-formed microspheres obtained from O/W emulsions with different PLGA, PRG, and PVA concentrations. *circle* FNº 7, *triangle* FNº 8, *square* FNº 9, and *inverted triangle* FNº 10. See Table 3 for more details

polymer/water interface during microparticle preparation [36, 37]. This fact, in addition to an increased viscosity of phase 2 (see Fig. 1), could explain the improved stability (>48 h) for systems with PVA and progesterone. The median size of microspheres increased with the increase of PLGA concentration at both PVA levels. The explanation could be an increment of phase 1 viscosity associated to higher concentrations of PLGA [10, 38]. During emulsification, the exposed time or rate of dispersing element (see "Preparation of Emulsions and In Situ Formed Microparticles" section) needed to obtain an equal size distribution might be higher when the viscosity of a solution is increased [34, 35].

The fact that progesterone concentration is up to 90 % of its saturation limit in phase 1 (all formulations were based on solutions, not suspensions) could explain the lower *Eni* used for 20 % PLGA compared with 10 % PLGA formulations (Table 3). An ANOVA analysis demonstrated that *Ee* decreased with the increment of PVA concentration at two PLGA levels ($P<0.05$) and could be associated with a lower retention capacity of progesterone within microparticles with sizes of <10 μm. It could be possible that the hormone was not inside but in the surface of the microsphere and was lost during washing procedures prior to its quantification. Furthermore, we found that the solubility of progesterone increased from approximately 40 to 75 ppm in aqueous solutions of 1 and 2 % of PVA, respectively (evaluated according to the methodology detailed in the "Solubility of Progesterone" section). During emulsification and microparticle precipitation, this higher solubility could cause greater diffusion of hormone from dispersed to continuous phase with increasing PVA content.

The instability of some characterized emulsions made them precipitate as agglomerates or large microparticles (Table 2). A stable emulsion can maintain its microglobules size and shape for a long time and during microparticles precipitation, but microglobules of an unstable emulsion flocculate and coalesce before or during microsphere precipitation, leading to higher microparticle sizes. Other phenomenon well documented in literature, could be distinguished: the slow diffusion of partially soluble solvent triacetin from emulsion to external aqueous media lead to PLGA concentration and shrink of microparticles during solvent exchange. Figure 2 shows that size distribution of microparticles from FNº 8 and 10 decreased compared with droplet size of its emulsion. Moreover, a SEM photomicrograph of FNº 10 (Fig. 3) shows that slow diffusion of triacetin and shrink also resulted in microspheres with a compact morphology, smooth surface, and without appreciable pores. These morphological characteristics caused by different rates of precipitation have been reported during in situ formation of implants [20, 22, 39] and microspheres [16, 31].

Figure 4 shows cumulative percentages of released progesterone against time plots of in situ-formed microspheres from formulations of Table 3. Narrowed error bars showed good reproducibility. The studied microspheres release a considerable quantity of hormone for the first 6 h. Taking into consideration that in vitro precipitation of PLGA occurs during the first part of this period (≤2 h), the burst effect observed could be associated in part to no-entrapped progesterone during hardening of phase 1 emulsions. After hardening of the emulsions, the release of the drug continues being fast. The fact that the magnitude of this burst release agrees with the *Ee* of each formulation presented in Table 3 (approximately 60–70 %), suggests that the drug released the first hours corresponds to drug weakly retained on the surface of the microspheres, the same drug that was lost in the microspheres washout protocol. Except for FNº 8, all the microspheres presented very similar release curves against time and released more than 95 % of initial progesterone load in 5 days. Microspheres from FNº 8 reached 100 % of released drug at the end of the assay, probably due to a fast release at the beginning (Fig. 4). Although the burst release effect is not desirable in a lot of

situations, it can be used to deliver drugs at high release rates as part of a drug administration strategy [40].

Results from this work demonstrate that triacetin/water emulsion was a useful solvent system to obtain microspheres capable to control-release progesterone for almost 5 days, in accordance with the hydrophobic nature of this hormone. In addition, PVA was successfully implemented to increase the stability of the O/W emulsions from 5 min to more than 48 h. This improvement in stability would ensure an effective and reproducible parenteral administration of studied formulations by advanced injection devices. These include solubilization and premix of components that guarantee robustness by generating a single dose a few minutes prior to its administration [28, 41]. However, the incorporation of these injection techniques could change the presented results and must be carefully studied. Finally, we recognize that in vivo release assays should be carried out to understand if the achieved rate and extent of progesterone release are acceptable.

Conclusions

In situ-formed microspheres are an alternative to expensive and complex manufactured preformed systems for the controlled release of drugs. We could demonstrate that emulsions based on PLGA 502H, triacetin and PVA aqueous solutions were useful formulations to obtain microparticles capable to controlled release progesterone for almost 5 days, in accordance with the hydrophobic nature of this hormone. In addition, the higher viscosity of phase 2 when PVA was implemented lead to more stable O/W emulsions compared with formulations using P188. In situ-formed microparticles based on stable O/W emulsions resulted to spherical and not aggregated polymeric systems after in vitro precipitation. Their compact morphology, smooth surface, and the absence of appreciable pores could be related to the slow diffusion of triacetin and shrinking of microparticles during in vitro precipitation.

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Conflict of interest The authors report no conflict of interest.

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