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Preparation and characterization of poly(lactic-co-glycolic acid) microspheres loaded with a labile antiparkinson prodrug

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ABSTRACT

L-Dopa-α-lipoic acid (LD–LA) is a new multifunctional prodrug for the treatment of Parkinson's disease. In human plasma, LD–LA catechol esters and amide bonds are chemically and enzymatically cleaved, respectively, resulting in a half-life time of about fifty minutes. In the present work, the unstable LD–LA was entrapped into biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres designed as depot systems to protect this prodrug against degradation and to obtain a sustained release of the intact compound. The microspheres were prepared by an oil-in-water emulsion/solvent evaporation technique and the effect of formulation and processing parameters (polymer concentration in the organic solvent, volumes ratio of the phases, rate of the organic solvent evaporation) on microspheres characteristics (size, loading, morphology, release) was investigated. Also emphasis was given on the stability of the drug before and after release as well as on the underlying mass transport mechanisms controlling LD–LA release. Interestingly, when encapsulated in appropriate conditions into PLGA microspheres, the labile prodrug was stabilized and released via Fickian diffusion up to more than one week.

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder which is characterized by the loss of dopaminergic neurons of the substantia nigra pars compacta with a subsequent reduction of dopamine (DA) neurotransmitter. PD affects about 1% of the population above the age of sixty, and its major clinical symptoms include movement disorders, anxiety, depression and dementia (Dauer and Przedborski, 2003; Duvoisin, 1987; Tanner, 1992; Gibb, 1992; Blandini and Greenamyre, 1999). The aetiology of PD has a multifactorial origin including oxidative stress and brain iron dysregulation (Dauer and Przedborski, 2003; Blandini and Greenamyre, 1999; Andersen, 2004; Ben-Shachar et al., 1991). The current chemotherapy is essentially symptomatic using exogenous L-Dopa (LD), the direct precursor of DA, as drug (Blandini and Greenamyre, 1999; Andersen, 2004; Fahn, 2006). LD administration is however associated with three important problems: (i) LD metabolism generates a variety of cytotoxic reactive oxygen species (ROS) that contribute to the progression of the disease; (ii) LD has a poor bioavailability in the CNS; (iii) during chronic treatment, patients become sensitive to LD plasma level fluctuations (Dauer and Przedborski, 2003; Bindoli et al., 1992; Di Stefano et al., 2001). In order to overcome these problems, a multifunctional prodrug (LD–LA, Fig. 1) containing LD and natural lipoic acid moiety (LA) has been recently synthesized (Di Stefano et al., 2006). Lipoic acid, which readily crosses the blood–brain barrier (BBB) and subsequently accumulates in neurons, has both antioxidant and iron-chelating properties. This means that it is active in reducing auto-oxidation of catecholamines (accelerated by transition metals) and in scavenging the ROS generated by LD metabolism (Ben-Shachar et al., 1991; Smith et al., 2004; Packer et al., 1997). LD–LA activity against Parkinson's disease symptoms and its antioxidant efficiency have been demonstrated using both in vitro and in vivo studies (Di Stefano et al., 2006). LD–LA therefore represents a good example of a multifunctional drug for treating central nervous system diseases. Moreover, in comparison with LD, oral administration of LD–LA prolonged the plasma LD concentration and this prodrug can therefore be particularly beneficial in the treatment of motor fluctuation, directly related to LD plasma level fluctuations. However, LD–LA catechol esters and amide bond are cleaved both chemically and enzymatically, resulting in a plasma half-life of about fifty minutes. Therefore multiple administrations per day are required to keep effective brain levels of LD (Di Stefano et al., 2006).

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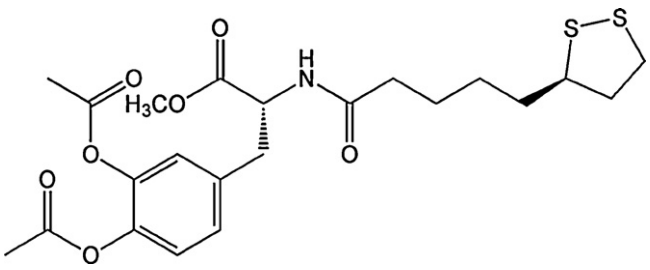


Fig. 1. LD–LA (methyl O-acetyl-3-(acetyloxy)-N-{5-[(3R)-1,2-dithiolan-3-yl]-pentanoyl}-L-tyrosinate) molecular structure.

In the present work, the labile prodrug LD–LA was entrapped into biodegradable polymeric microspheres to protect this prodrug from enzymatic and chemical degradation and to obtain a sustained release of the intact compound. Poly(lactic-co-glycolic acid) (PLGA), was used to prepare prodrug-loaded microparticles because this polymer has a good biocompatibility and is biodegradable (Anderson and Shive, 1997; Fournier et al., 2003; Jain, 2000; De Luca et al., 1993; Varde and Pack, 2004). Therefore it has been used for the development of sustained drug formulations of low molecular weight compounds as well as therapeutic proteins and plasmid DNA (Varde and Pack, 2004; Giovagnoli et al., 2008; Fernández-Carballido et al., 2004; Elkharraz et al., 2006; Janoria and Mitra, 2007; Ye et al., 2010; Jiang et al., 2005; Abbas et al., 2008). Due to the lipophilic character of LD–LA, PLGA microspheres were formulated using an oil-in-water emulsion/solvent evaporation method (Jain, 2000; Wischke and Schwendeman, 2008). The effect of formulation and processing parameters on the microspheres properties (size, loading, release) was investigated. Also emphasis was given on the stability of the drug before and after release as well as on the underlying mass transport mechanisms controlling LD–LA release.

2. Materials and methods

2.1. Materials

PLGA 50:50 Purasorb 5004A (intrinsic viscosity 0.41 dL/g) with free carboxyl end-groups was obtained from Purac Biomaterials. Polyvinyl alcohol (PVA, degree of hydrolysis 88%, molecular weight ranging from 13,000 to 23,000) was purchased from Aldrich Chemical Company. HPLC grade dichloromethane (DCM) and methanol were obtained from Biosolve BV. Dimethyl sulfoxide (DMSO, absolute) was obtained from Fluka. Formic acid, acetic acid anhydrous, NaOH, NaH_2PO_4 monohydrat, Na_2HPO_4 dihydrat, and NaCl (all pro-analysi grade) were obtained from Merck. NaN_3 (99.5%) was obtained from Sigma. The LD–LA prodrug was synthesized as previously described (Di Stefano et al., 2006).

2.2. Preparation of the microspheres

LD–LA loaded PLGA microspheres were prepared by an oil-in-water emulsion/solvent evaporation technique (o/w, ESE) (Wischke and Schwendeman, 2008). Briefly, 0.5 mL of oil phase (o-ph) was prepared by dissolving both PLGA and LD–LA in DCM in different concentrations and drug/polymer ratios (200, 400, 800 mg of PLGA per mL of DCM; drug/polymer ratio of 2%, 5% or 20% (w/w)). For the o-ph with the higher polymer concentration, the solutions were sonicated for 30 min at room temperature. The o-ph was emulsified (30 s, room temperature, 25000 min^{-1}) with 0.5, 2.5 or 5 mL of aqueous phase (aq-ph) consisting of PVA 2% (w/w) in water (previously saturated, or not, with DCM) by using an IKA Ultra-Turrax T8 homogenizer. In order to allow DCM evaporation and microsphere solidification, the resulting emulsion was then added drop wise to a PVA aqueous solution (0.5% (w/w); 2.5 or 5 mL), the hardening bath (hb), and magnetically stirred for two hours at room temperature. Alternatively, the emulsion was stirred without dilution (Table 1). The microspheres were collected by centrifugation at room temperature (3 min, 3000 × *g*), subsequently washed twice with water, flash frozen with liquid N_2 and freeze-dried overnight.

2.3. Microspheres characterization

The size and size distribution of the microspheres were measured using an Accusizer 780 (Optical particle sizer, Santa Barbara, California, USA). Scanning electron microscopy (S.E.M.) using a Phenom (FEI Company, The Netherlands) microscope was employed to study the size, porosity and morphology of the microspheres. Modulated differential scanning calorimetry (DSC) analyses were performed on prodrug, on empty and on prodrug loaded microspheres by means of a TA Instruments DSC Q2000. About 5 mg of material was loaded into aluminium pans, which were sealed, and subsequently equilibrated at −20 °C for 5 min. They were then heated from −20 to 120 °C at a heating rate of 1 °C/min, subsequently cooled to −20 °C (5 °C/min) and then heated again to 120 °C (1 °C/min). The glass transition temperature (T_g) was determined from the thermogram recorded during the second heating cycle.

The drug loading of the microspheres was determined by HPLC quantification of the prodrug extracted from the microspheres. Briefly, 5 mg of dried microspheres was dissolved in 0.5 mL of DMSO, followed by the addition of 1 mL of cold methanol to precipitate the PLGA. Next, the samples were centrifuged (30 min, 20,000 × *g*, 4 °C), the supernatants were filtered and methanol was evaporated under vacuum to obtain DMSO solutions of the

Table 1
Characteristics of the different prodrug loaded microspheres formulations. Data are expressed as mean ± SD ($n = 3$), hb denotes hardening bath.

Formulation	PLGA concentration in o-ph (mg per DCM mL)	DCM pre-saturation of aq-ph	Phases volume ratio (o-ph/aq-ph/hb)	TDL (%)[a]	EDL (%)[b]	LE (%)[c]	Volume–weight diameter (μm)
F1	200	No	1/1/10	2	1.5 ± 0.2	59 ± 3	16 ± 8
F2	200	No	1/1/10	5	3.2 ± 0.3	46 ± 2	11 ± 6
F3	200	No	1/1/10	20	14 ± 1	46 ± 5	13 ± 9
F4	400	No	1/1/10	5	3.4 ± 0.2	47 ± 2	30 ± 13
F5	400	No	1/1/10	20	14 ± 1	37 ± 3	21 ± 11
F6	400	Yes	1/5/5	5	3.2 ± 1	50 ± 5	34 ± 18
F7	400	Yes	1/10/0	5	2.7 ± 0.9	37 ± 8	30 ± 15
F8	800	Yes	1/5/5	5	2.7 ± 0.1	25 ± 1	26 ± 20
F9	800	Yes	1/10/0	5	2.6 ± 0	15 ± 8	28 ± 23

[a] TDL (theoretical drug loading) expressed as ratio between mass of drug and mass of polymer used in formulation.
[b] EDL (effective drug loading) expressed as mass ratio of drug entrapped in microspheres.
[c] LE (loading efficiency) expressed as mass ratio of drug entrapped in microspheres and drug used to prepare them.

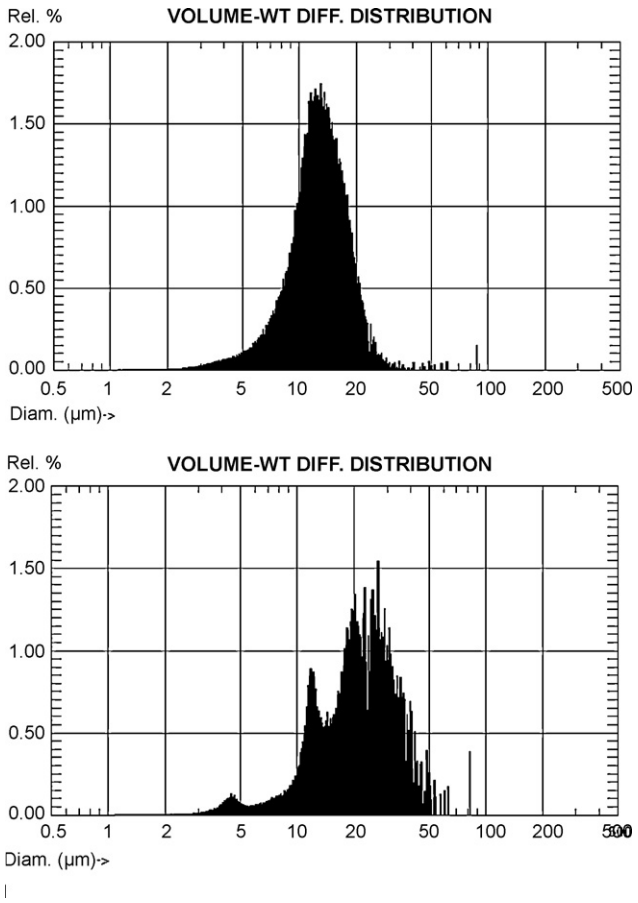


Fig. 2. Representative examples of size distribution results (volume-weight mean diameter) obtained by Accusizer measurements. Top: F2 formulation (PLGA in DCM 200 mg/mL in the o-ph). Bottom: F8 formulation (PLGA in DCM 800 mg/mL in the o-ph).

prodrug. The LD–LA concentration in the DMSO solutions was measured using a Waters 2695 separations module equipped with a Waters 2487 UV-detector and a Waters SunFire C18 RP column (4.6 mm × 150 mm, 5 μm). A pH 4.5 buffered methanol:water (60:40) mixture was used as eluent (flow rate 0.5 mL/min, detection wavelength 230 nm). Calibration was done with prodrug dissolved in DMSO. This HPLC method allows separation of intact LD–LA from its degradation products (see below). Control experiments (LD–LA was extracted from physical mixtures of drug and polymer as described above) demonstrated that the drug was quantitatively recovered. Further, it was shown that no detectable prodrug hydrolysis occurred during sample preparation and analysis.

2.4. In vitro release studies

Drug release of PLGA loaded microspheres was monitored at 37 °C, under constant shaking and sink conditions, in buffers of pH 4.5 and 7.4. Microspheres (5 mg) were suspended in 1.5 mL of release buffer and incubated while gently shaken. After centrifugation, the supernatants were removed completely and replaced with fresh buffer solutions. The drug release in pH 4.5 acetate buffer (0.02 M acetic acid, 0.012 M NaOH) was monitored by filtering the release samples through 0.2 μm filters (Grace Davison Discovery Science) and analysing the filtrates with the HPLC method described in Section 2.3. Calibration was done with LD–LA dissolved in pH 4.5 acetate buffer.

The drug release in pH 7.4 phosphate buffer (PBS, 0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05% (w/w) NaN_3), was determined as follows. Since at this pH the released drug undergoes degradation, making quantification of the amount of drug released difficult, we determined the intact, non-released prodrug in the microspheres. In detail, the microspheres were separated from the buffer by centrifugation, washed twice with water, flash-frozen in liquid nitrogen and freeze-dried overnight. The drug content of the microspheres was determined by the same method as described in Section 2.3.

2.5. Drug hydrolysis

The LD–LA chemical hydrolysis at pH 4.5 and pH 7.4 was studied at 37 °C by means of HPLC–MS analysis. LD–LA was first dissolved in methanol (5 mg/mL) and 1.2 μL of this solution was diluted both in PBS and in acetate buffer (composition see Section 2.4) to a final volume of 2.0 mL to obtain a final concentration of 30 μg/mL. At different time points, 20 μL of each sample was analyzed using the HPLC method described in Section 2.3. The eluent was buffered at pH 4.5 with formic acid and NaOH. Calibration was done with solutions of LD–LA in the same medium. The separation of the intact compound from its decomposition products was achieved by a Waters SunFire C18 RP column. The identity of the different degradation products was established using LC–MS by means of an SCL-10A VP system controller, an LC-10AD VP liquid chromatograph, a column oven CTO-10AS VP and an SPD-10A VP UV–vis detector (Shimadzu Scientific Instruments) connected with a Finningan LCQ Deca XP MAX ion trap mass spectrometer equipped with an electron spray ionization source (Thermo Electron Corporation).

3. Results and discussion

3.1. Preparation and characterization of LD–LA loaded PLGA microspheres

LD–LA loaded PLGA microspheres were prepared by an oil-in-water emulsion/solvent evaporation technique (o/w ESE), a frequently used method to encapsulate hydrophobic compounds that like LD–LA are highly soluble in volatile organic solvents (Wischke and Schwendeman, 2008). PVA was used as an emulsifier. It is known that a fraction of PVA (the preferred stabilizer for the preparation of PLGA formulations) adsorbs onto the microspheres surface and cannot be completely removed despite washing because PVA forms an interconnected network with the polymer at the interface (Alléman et al., 1993; Sahoo et al., 2002). Although PVA is not biodegradable, it is water-soluble and its renal excretion can be expected when the molecular mass is below 40 kDa (Besheer et al., 2007; De Merlis and Shoneker, 2003), like in this case (PVA used had an average molecular weight of 13–23 kDa).

The effect of formulation parameters (polymer concentration in the organic solvent, volumes ratio of the phases, rate of the organic solvent evaporation) on microsphere characteristics (size, loading efficiency, morphology) was evaluated (Table 1). The microspheres were obtained in a good yield (usually >70%). Table 1 shows that an increase in PLGA concentration resulted increasing microspheres sizes and polydispersity (for example compare F2 and F4). These phenomena can be ascribed to an increase of the viscosity of the oil phase that results in a reduction of the stirring efficiency, which in turns yields polydispersed and larger emulsified droplets and consequently larger microspheres with a rather broad size range (Zhu et al., 2003; Ghassemi et al., 2009). Moreover, it was shown by Rosca et al. (2004) that the shrinkage factor (the ratio between the diameter of the emulsion droplets and the diameter of the solvent-free microparticles) is larger for lower polymer concentrations, contributing to a further decrease of the microspheres size.

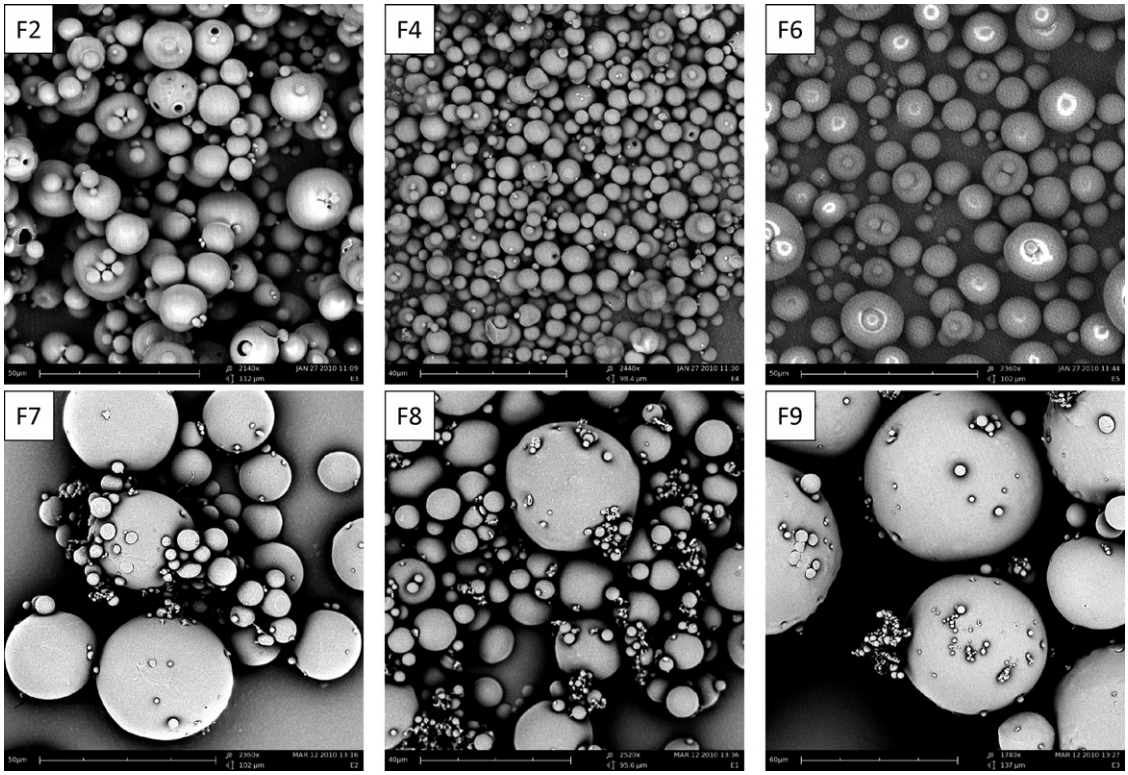


Fig. 3. S.E.M. images of F2, F4, F6, F7, F8 and F9 microspheres formulations.

In order to obtain injectable microparticles for depot applications, a particle size range of 20–100 μm is usually desired (Wischke and Schwendeman, 2008), which goal was reached for all the formulations. Polydispersity of microspheres is invariably associated with emulsion-based preparation techniques (Jalil and Nixon, 1990; Leach et al., 2005). Accusizer measurements (Fig. 2) showed that the microspheres prepared using a DCM solution of 200 mg PLGA/mL had a rather narrow size distribution, whereas at increasing polymer concentration (800 mg/mL) the particle size range broadened.

S.E.M. analysis shows that an increase in polymer concentration affected also the morphology of the microspheres. Microparticles prepared using 200 mg PLGA/mL DCM (Fig. 3, F2) had holes and some particles were even collapsed, whereas microparticles prepared using the double concentration were considerably less porous and no collapsed structures were observed (Fig. 3, F4). Presaturation of the aq-ph with DCM, which reduces the rate with which DCM is extracted into the aqueous phase and thus the rate of particle solidification (Wischke and Schwendeman, 2008; Rosca et al., 2004; Soppimath and Aminabhavi, 2002), also resulted in smooth, non porous particles (Fig. 3, F6 and F7). Particles prepared using a DCM solution with a high PLGA concentration (800 mg/mL) were non-porous, but, in agreement with Accusizer data (Fig. 2) showed a rather broad size distribution (Fig. 3, F8).

The LD–LA loading efficiency was around 50% (except formulations F8 and F9) and not substantially influenced by formulation parameters. Fig. 4 shows the thermograms of the LD–LA, the empty particles and the drug-loaded particles (formulations F4 and F5, effective drug loading 3.4% and 14%, respectively). Single T_g's are observed in the four thermograms. Interestingly, the T_g's of the LD–LA loaded microspheres are between that of the empty microspheres (47.3 °C) and that of LD–LA (1.5 °C), and perfectly matches the value calculated using the Fox's equation (Gedde, 1995):

$$\frac{1}{T_g} = \sum \left(\frac{w_i}{T_{gi}} \right) \quad (1)$$

where T_g is the glass transition temperature of a miscible polymeric blend or a plasticized polymer (in K), T_{gi} indicates that of the component i of the mixture and w_i is its weight fraction. The good agreement between the observed T_g and the one calculated using Fox's equation means that LD–LA is molecularly dispersed in the PLGA microspheres, at least up to a prodrug amount of 14% (Gedde, 1995; Blasi et al., 2007; Hombreiro-Pérez et al., 2003). Additional DSC analyses were performed on the other formulations, with analogous results (see Supplementary data section).

3.2. LD–LA chemical hydrolysis

As mentioned in Section 1, LD–LA is susceptible to both chemical and enzymatic degradation (Scheme 1). Degradation can occur during preparation of the microspheres and during storage, whereas

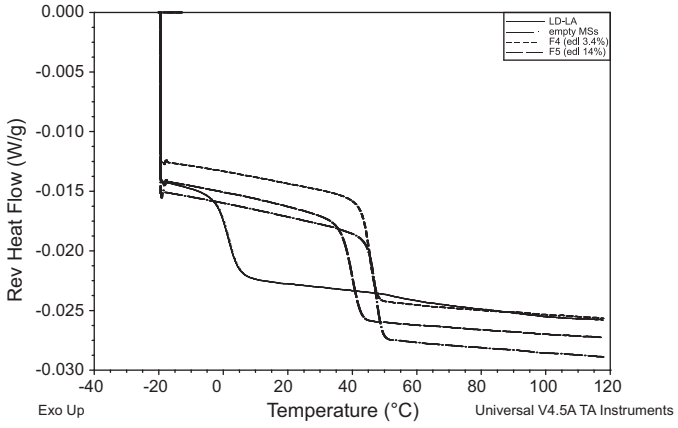
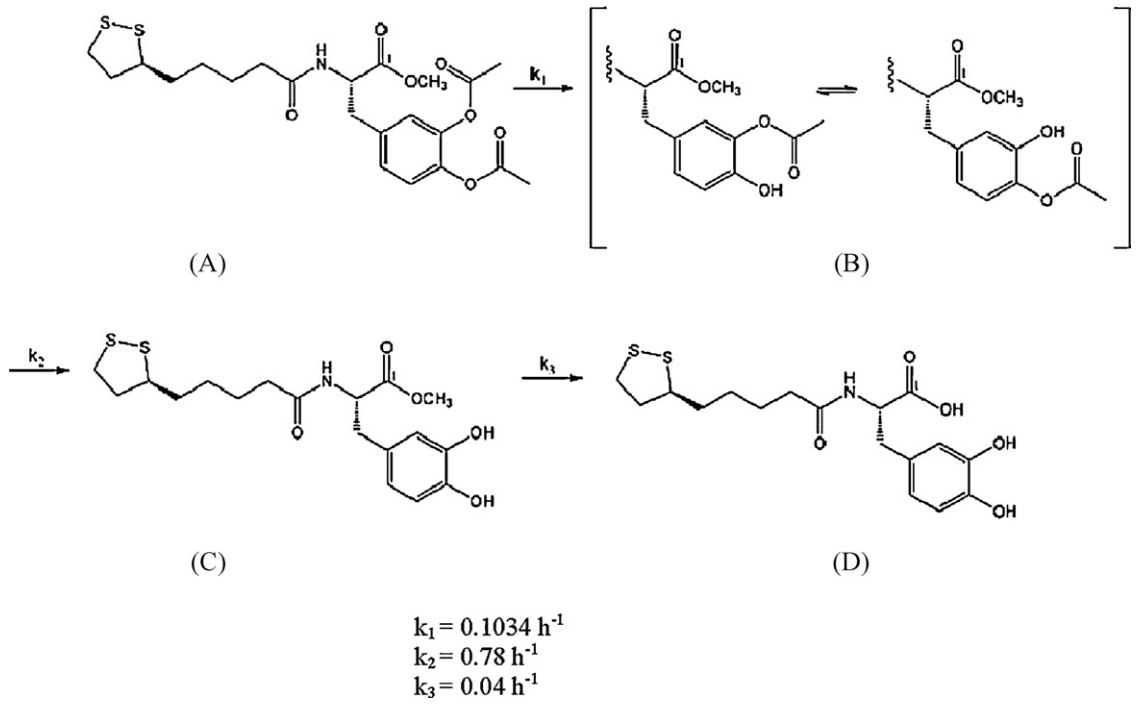


Fig. 4. Thermograms of LD–LA (T_g = 1.5 °C), prodrug loaded microspheres with 14% (F5, T_g = 39.7 °C), 3.4% (F4, T_g = 45.3 °C) of EDL, and empty microspheres prepared in the same conditions (T_g = 47.3 °C).



Scheme 1. LD–LA multistage chemical hydrolysis (kinetic constants values are for the hydrolysis in PBS at 37 °C). The intermediate B, resulting from the first deacetylation at the catecholic moiety, is supposed to exist as mixture of two isomers because of the rapid and reversible O → O acyl migration between the neighbor hydroxyl groups that occurs in solution (Ihara et al., 1990).

LD–LA can also degrade in the particles once incubated at 37 °C in an aqueous buffer and after being released. The drug loading was determined by HPLC analysis as described in Section 2.3. The retention time of the intact prodrug was 20 min, while its degradation products had shorter retention times. However, no other peaks in the chromatograms were detected, demonstrating that no degradation of LD–LA occurred during preparation and storage at room temperature for 1–4 weeks of the microspheres (data not shown).

The LD–LA degradation kinetics were studied using LC–MS analysis. The intact prodrug (A) undergoes a base-catalyzed multistage degradation process. Firstly, it is deacetylated at the catecholic moiety with first-order kinetics to yield intermediate B that subsequently, more rapidly [because of the stabilization of the transition state by the formed OH group and because of the less steric hindrance (Ihara et al., 1990)], degrades into product C (still able to cross the BBB and pharmacologically active). This last compound then degrades into a not identified product D, probably formed by hydrolysis of the methyl ester at position 1 (Scheme 1). The degradation of LD–LA was studied by incubation of this compound at pH 7.4 and 4.5, and at 37 °C. At pH 4.5 no detectable degradation of LD–LA was observed during 48 h of incubation. At pH 7.4 however, LD–LA rapidly degraded. To determine the kinetic constants of the different reactions, the concentration of the different compounds is plotted against time (Fig. 5). The monoacetyl degradation product B is in equilibrium with an isomer formed by acyl migration. The conversion of the one isomer into the other is so rapid (Ihara et al., 1990) that the two isomers elute as a single entity. The decrease in prodrug concentration [A] follows first order kinetics:

$$\ln[A]_t = -k_1 \cdot \ln[A]_0 \quad (2)$$

where $[A]_t$ and $[A]_0$ are the concentrations of A at time t and at time zero, respectively and k_1 is the first order reaction constant, calculated from the slope of the ln[A] versus time plot. k_2 was calculated from the time point (see Fig. 5) at which the concentration of the monoacetyl intermediate, [B], reached its maximum value and a plateau ('steady state'). At that time point the rate of formation of B equals the rate of degradation of B, (or: $k_1[A] = k_2[B]$). Thus, from the calculated k_1 and experimental [A] and [B] at that time point, k_2 was calculated. A plateau in the second degradation product concentration [C] can be also observed. Therefore, a steady state situation exists, i.e. $k_2[B] = k_3[C]$, and k_3 can be calculated. In Scheme 1, the calculated values for k_1–k_3 are given, corresponding with a half life of 6.7, 0.89 and 17 h at pH 7.4 and 37 °C for compound A, B and C, respectively. In a previous study we have demonstrated that the degradation products B and C are still pharmacologically active, but because of a higher hydrophilicity they are less able to pass the BBB and they are more susceptible for enzymatic degradation (Di Stefano et al., 2006). To verify the reliability of the calculated constants, they were put in the following equations describing the concentration of compounds A, B and C assuming two consecutive first-order reactions (Atkins, 1997):

$$[A]_t = [A]_0 e^{-k_1 t} \quad (3)$$

$$[B]_t = [A]_0 \left(\frac{k_1}{k_2 - k_1}\right)(e^{-k_1 t} - e^{-k_2 t}) \quad (4)$$

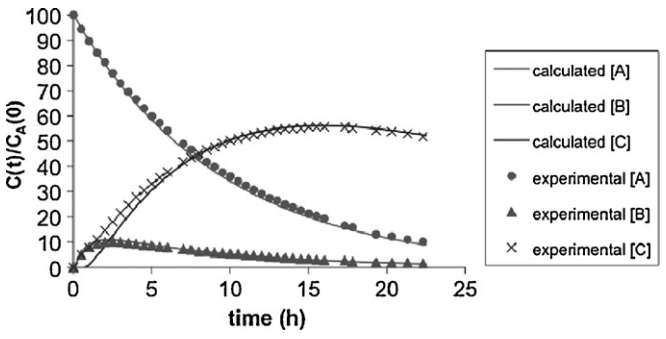


Fig. 5. Fitting of the theoretical curves (solid lines) to experimental data of relative concentrations (ratio between concentration at time t and concentration of starting compound A at time zero) of intact prodrug A and its first (B) and second (C) hydrolysis products during time.

$$[C]_t = [A]_0 \left\{ 1 + \left(\frac{1}{k_1 - k_2} \right) (k_2 \cdot e^{-k_1 t} - k_1 \cdot e^{-k_2 t}) \right\} \quad (5)$$

where $[A]_0$ is the concentration of the intact compound A at time zero and $[A]_t$, $[B]_t$ and $[C]_t$ are the concentrations at time t of compounds A, B and C, respectively. An excellent fit of the experimental data is found (Fig. 5).

3.3. In vitro drug release

In the previous section it was demonstrated that the LD–LA prodrug is rather unstable at pH 7.4, but has a good stability at pH 4.5. Normally, release studies with drug-loaded PLGA systems are performed at pH 7.4 (Fernández-Carballido et al., 2004; Janoria and Mitra, 2007; Ghassemi et al., 2009). But, because in buffer of pH 7.4 the drug will degrade, we cannot distinguish whether or not the drug undergoes (unwanted) degradation in the hydrated PLGA microspheres or in the bulk fluid. Therefore, we selected the following experimental set up for getting insight into the release properties of the LD–LA loaded microspheres and the stability of the prodrug in the hydrated PLGA particles. The microspheres were incubated in a pH 7.4 buffer and the remaining (non-released) amount of prodrug in the microspheres was measured as a function of time by dissolving the particles in DMSO. Also, the release was followed at pH 4.5 by measuring the LD–LA concentration in the buffer at pre-determined time points. It was found that no degradation of the prodrug in the PLGA particles occurred upon incubation for 260 h at 37 °C. Likely, although the PLGA particles absorb some water [less than 15% (Shenderova et al., 1999)], the water activity is obviously too low to induce chemical hydrolysis of the ester bonds in the prodrug. Also, the low pH generated in the matrix due to the COOH end-groups of the polymer (Sophocleous et al., 2009; Wong-Moon et al., 2008) might be responsible for stability of the prodrug. Importantly, it was found that the amount of intact prodrug present in the microspheres incubated in pH 7.4 buffer at time t (Q_{in}) equaled the difference between the loaded drug amount (Q_{enc}) and the drug amount released at time t from the same microsphere formulations suspended in pH 4.5 buffer (Q_{out}):

$$Q_{in}(t) = Q_{enc} - Q_{out}(t) \quad (6)$$

It can therefore be concluded that: (i) LD–LA is not degraded while entrapped inside the microspheres and (ii) there is no significant difference in drug release from these formulations between pH 4.5 and pH 7.4. The release of LD–LA from the microspheres demonstrated to be fast compared to the degradation time of PLGA matrices in microspheres (Anderson and Shive, 1997). In this short period a potential impact of the pH of the release medium on polymer matrix erosion and drug release seems to be negligible.

Fig. 6 shows the experimentally measured release profiles of LD–LA from the different microsphere formulations. The following analytical solution of Fick's second law of diffusion was used to describe LD–LA release from the investigated PLGA-based microspheres. This model considers the spherical geometry of the systems, homogeneous initial drug distribution, perfect sink conditions during the release experiment and the fact that LD–LA is molecularly distributed within the polymeric matrix (monolithic system). It is based on the assumption that drug release is purely controlled by diffusion with constant diffusivities (Faisant et al., 2002; Arifin et al., 2006):

$$\frac{M_\infty - M_t}{M_\infty} = \frac{6}{\pi^2} \cdot \sum_{n=1}^{\infty} \frac{1}{n^2} \cdot \exp\left(-\frac{n^2 \cdot \pi^2}{R^2} \cdot D \cdot t \right) \quad (7)$$

where M_∞ and M_t denote the absolute cumulative amounts of drug released at infinite time and time t, respectively; R represents the average radius of the microspheres and D the apparent diffusion coefficient of the drug in the PLGA matrix (neglecting size-distribution effects). The curves in Fig. 6 show fittings of this equation to the experimentally measured drug release kinetics (symbols). Based on these calculations, the apparent diffusion coefficient of LD–LA in each formulation could be determined (Table 2). The good agreement between the applied theory and the experimental data for the different microsphere formulations indicates that pure diffusion is likely to be the dominant drug release mechanism for all formulations. The drug release profiles of formulations F7, F8 and F9 were very similar and similar apparent diffusion coefficients were determined (Table 2). This is sound, since the average microparticle sizes were in the same range for these formulations (Table 1). In contrast, F2, F4 and F6 microparticles showed faster drug release rates (Fig. 6). This is likely to be attributable to the porosity of these systems, which were prepared using less concentrated PLGA solutions in DCM (even though F6 microparticles mainly show a non-porous outer surface, probably because of the too low magnification of S.E.M. pictures (Rosca et al., 2004)). The surface porosity of PLGA microspheres prepared by o/w single emulsion method depends indeed on the extent of shrinkage of the emulsion oil-droplets due to DCM evaporation from the forming microspheres, and the extent of shrinkage is significant during the intense solvent elimination from initial droplets with low polymer concentration (Rosca et al., 2004). In the case of F2 microspheres, also the small size of the systems (Table 1) explains the observed rapid drug release rate: the shorter diffusion pathway lengths even overcompensate the lower apparent LD–LA diffusivity compared to formulations F4 and F6 (Table 2). The fact that F6 microparticles released the drug more slowly than F2 or F4 formulations (especially at early times) might be attributable to the less porous surface of the systems (Fig. 3).

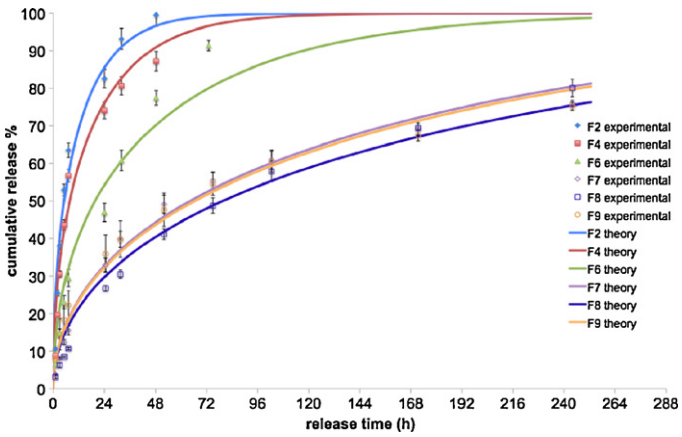


Fig. 6. In vitro LD–LA release profiles from the different formulations studied: experiment (symbols) and theory (curves, Eq. (7)).

It can be concluded that formulation parameters strongly affect the microspheres' characteristics (including the drug release rate), in particular observing the differences between formulations F6 and F7. They were prepared using the same polymer concentration in the oil-phase, but different phase volume ratios (affecting

Table 2
Effects of the type of microspheres on the apparent diffusion coefficient D of LD–LA within the system.

Formulation	D (10^{-12} cm^2/s)	R^2
F2	0.51	0.99
F4	2.50	0.99
F6	1.25	0.98
F7	0.30	0.98
F8	0.18	0.98
F9	0.25	0.99

the rate of DCM extraction and subsequent microspheres solidification): probably the faster solidification of F6 microspheres led to a less dense polymeric matrix compared to formulation F7 (Soppimath and Aminabhavi, 2002) and so to a faster release of the drug. This is in good agreement with the higher apparent diffusion coefficient of the drug in formulations F6 versus F7 (Table 2). Analogously, the effect of the polymer concentration of the oil-phase on drug release becomes obvious when comparing formulations F6 and F8: they were both prepared with the same phase volume ratio and using aqueous phases saturated with DCM, but F8 was prepared using twice the concentration of PLGA (800 mg/mL). Clearly, F8 released the drug more slowly than F6 (400 mg PLGA/mL) and had an apparent diffusion coefficient, which was smaller than that of F6 microparticles. The higher polymer concentration is likely to result in smoother and denser polymeric structures and, thus, more pronounced hindrance for LD–LA diffusion.

4. Conclusions

The antiparkinson prodrug LD–LA can be loaded with high efficiency into PLGA microspheres made by o/w ESE method. Analyses of microspheres content did not reveal degradation of the loaded prodrug neither during microspheres preparation, nor during storage of the dried formulations, nor once they were incubated in release conditions. Probably thanks to its acidic micro-climate, the PLGA microsphere matrix was able to protect the dissolved prodrug from the chemical hydrolysis it undergoes under physiological conditions. For all the formulations prepared the microspheres size was between 20 and 100 μm, like desired for injectable depot applications. Critical formulation and processing parameters strongly affected size and porosity of microspheres, with consequent influence on drug release rate. In particular, the polymer concentration in the organic phase, the phases volume ratio and the pre-saturation of the aqueous phase with the organic solvent have an important effect on stirring efficiency and oil-droplets solidification rate, and in consequence on microspheres size, polydispersity and porosity. DSC studies indicated that the prodrug was molecularly dissolved in the polymeric matrix of the microspheres, up to a concentration of 14% (w/w). Prodrug release is basically governed by diffusion and can be tailored by controlling the above mentioned formulation and processing parameters, so that a sustained prodrug release via Fickian diffusion can be obtained up to more than one week from microspheres formulated under appropriate conditions. Future studies should be done in order to verify if the sustained release properties observed in vitro could result in an in vivo continuous dopaminergic stimulation, which is the needed condition to limit the motor complications characterizing Parkinson's disease.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.02.036.

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