



PLGA microparticles with zero-order release of the labile anti-Parkinson drug apomorphine

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ABSTRACT

The treatment of patients suffering from advanced Parkinson's disease is highly challenging, because the efficacy of the "gold standard" levodopa declines with time. Co-administration of the dopamine receptor agonist apomorphine is beneficial, but difficult due to the poor oral bioavailability and short half-life of this drug. In order to overcome these restrictions, PLGA-based microparticles allowing for controlled parenteral delivery of this morphine derivative were prepared using (solid-in-)oil-in-water extraction/evaporation techniques. Particular attention was paid to minimize spontaneous oxidation of the labile drug and to optimize the key features of the microparticles, including encapsulation efficiency, initial burst release and particle size. Various formulation and processing parameters were adjusted in this respect. The systems were thoroughly characterized using SEM, EDX, DSC, laser diffraction, headspace-GC as well as in vitro drug release measurements in agitated flasks and flow-through cells. Importantly, apomorphine could effectively be protected against degradation during microparticle preparation and within the delivery systems upon exposure to phosphate buffer pH 7.4 (containing 0.2% ascorbic acid) at 37 °C: 90% intact drug was released at a constant rate during about 10 d.

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1. Introduction

Apomorphine is a non-narcotic derivative of morphine, which can cross the blood brain barrier. It is used for the treatment of patients with advanced Parkinson's disease (Chen and Obering, 2005; Stacy and Silver, 2008; Goole and Amighi, 2009). The symptoms of this disorder are related to the degeneration of dopaminergic neurons in the substantia nigra and a reduction in the amount of the neurotransmitter dopamine available in the striatum (Dauer and Przedborski, 2003). Current treatment methods include: (i) dopamine substitution via administration of its metabolic precursor levodopa ("gold standard" therapy) (in contrast to dopamine, levodopa is able to cross the blood brain barrier). However, levodopa undergoes extensive decarboxylation during its passage through the liver (which might be reduced by co-administration of decarboxylase inhibitors, Barbeau et al., 1972), (ii) drugs, which inhibit the metabolism of dopamine, and (iii)

activation of striatal dopamine receptors by dopamine agonists. Apomorphine falls in this third category.

However, none of these therapeutic strategies is able to alter the progression of Parkinson's disease and with time the efficiency of levodopa decreases: Its doses must be increased and the duration of its action is reduced. Systemic side effects limit the maximum possible levodopa dose, and at advanced stages patients experience "on" and "off" phases, despite optimized levodopa therapy. "On" phases are periods with symptomatic improvements. "Off" phases are characterized by the return of motor features. They might present end-of-dose levodopa episodes or might be unpredictable. Such end-of-dose effects concern 30–50% of the patients within 5 years of starting levodopa therapy (Pahwa and Lyons, 2004). They often occur the day before the next dosing or in the mornings. The administration of a dopamine receptor agonist can help reducing these "off" phases in patients with advanced Parkinson's disease. Due to its structural similarities with dopamine, apomorphine is a highly potent dopamine receptor agonist: It has a similar affinity to D1, D2 and D3 receptors as dopamine (Factor, 1999; Newman-Tancredi et al., 2002). However, the administration of apomorphine is challenging: upon oral administration the drug is extensively degraded by hepatic first pass metabolism. To avoid this phenomenon, apomorphine can be injected subcutaneously

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(LeWitt, 2004). It has been shown that subcutaneous apomorphine injections can reduce the “off” times in patients treated with levodopa by 50–60% and that the levodopa dose can be reduced by 20% (Stibe et al., 1988). In 2004, subcutaneous apomorphine administration was approved as a rescue medication for patients with advanced Parkinson’s disease in the US. But apomorphine is unstable, systemic oxidation appears to be the major metabolic pathway (about 90% of the drug is likely to undergo auto-oxidation) (Van der Geest et al., 1997). The elimination half-life is only about 30 min (Gancher et al., 1989) and frequent injections or continuous infusion is required. Thus, parenteral controlled delivery of apomorphine, assuring efficient drug protection and optimized concentrations at the site of action during prolonged periods of time are likely to be able to offer major advantages (Slotty et al., 1995; Raasch et al., 2000; Hwang et al., 2009).

Poly(lactic-co-glycolic acid) (PLGA) based microparticles are well established as advanced drug delivery systems, allowing for drug protection and controlled release upon parenteral administration (Wischke and Schwendeman, 2008; Schoenhammer et al., 2009; Makadia and Siegel, 2011). PLGA is completely biodegradable (Anderson and Shive, 1997), biocompatible (Middleton and Tipton, 2000) and several products based on this polymer have been approved by the FDA for parenteral use (e.g. Rawat et al., 2012). PLGA has been shown to protect labile drugs, such as 9-cis-retinoic acid (Cosco et al., 2011). Furthermore, PLGA-based microparticles can deliver a wide range of drugs, controlling their release rates over periods ranging from a few days up to several months (Freiberg and Zhu, 2004; Rawat and Burgess, 2011; Fredenberg et al., 2011; Gaignaux et al., 2012). Compared to implants, they can generally be administered more easily. Different techniques can be used to prepare drug-loaded, PLGA-based microparticles, including spray-drying (Jain, 2000; Darbandi and Zandkarimi, 2012), coacervation (Thomasin et al., 1998), film grinding (Kranz and Bodmeier, 2007), spray congealing (Lee et al., 1997), and emulsion/dispersion solvent extraction/evaporation methods (Freitas et al., 2005). In this study, (solid-in-)oil-in-water solvent extraction/evaporation technique were applied. The impact of various processing and formulation parameters on the key properties of the microparticles has been reported in the literature, including the effects of the type of organic phase (Yang et al., 1999; Birnbaum et al., 2000), drug loading (Choi et al., 2002), pH of the outer aqueous phase (Mao et al., 2008), addition of NaCl or ethanol to the external aqueous phase (Luan et al., 2006), stirring rate (Zhang et al., 2008), volumes of the inner and outer phases (Li et al., 1995; Choi et al., 2002), and surfactant concentration in the outer aqueous phase (Yang and Owusu-Ababio, 2000).

The aim of this study was to encapsulate the labile drug apomorphine within PLGA-based microparticles to allow for: (i) an efficient protection against degradation during storage and upon exposure to aqueous media prior to release, and (ii) controlled release at a constant rate. Importantly, apomorphine is rapidly degraded in aqueous solution by oxidation (Kaul and Brochmann-Hanssen, 1961; Burkman, 1965) and is sensitive to light and oxygen (Garrido et al., 2002; Olea-Azar et al., 2002), even in the presence of antioxidants (Wilcox et al., 1980; Ng Ying Kin et al., 2001; Li et al., 2001). Thus, special precautions had to be taken during microparticle preparation.

2. Materials and methods

2.1. Materials

D,L-Lactide and glycolide (Purac, Gorinchem, The Netherlands) were recrystallized in toluene. Mesitylene (99%; Acros Organics, Geel, Belgium) was dried over molecular sieves (4 Å, powder;

Aldrich, Saint-Quentin-Fallavier, France) until the residual amount of water was below 5 ppm. Benzyl alcohol (99+%) (Aldrich, Saint-Quentin-Fallavier, France) was dried over sodium and distilled before used. Tin octanoate (Aldrich), poly(D,L-lactic-co-glycolic acid) (PLGA; Resomer RG 502H; PLGA 50:50; Boehringer Ingelheim, Ingelheim, Germany), R-apomorphine HCl (Francopia, Paris, France), acetonitrile and dichloromethane (HPLC Grade; Fisher Scientific, Loughborough, UK), polyvinyl alcohol (Mowiol 4-88; Sigma-Aldrich, Steinheim, Germany), ascorbic acid (Cooper, Melun, France), ethylenediaminetetraacetic acid (EDTA; VWR, Haasrode, Belgium), sodium chloride (Fisher Scientific), sodium sulfite (Acros Organics) and Tris Pufferan (Carl Roth, Karlsruhe, Germany) were used as received.

2.2. Synthesis of apomorphine ascorbate

An aqueous solution of sodium sulfite (10 mL, 16.4 mmol) was added dropwise onto a well-stirred aqueous solution of apomorphine HCl (150 mL, 6.58 mmol). The heterogeneous mixture was stirred for additional 30 min. Then, the free base apomorphine was extracted into diethyl ether (three times: 150, 50 and 50 mL). The organic extracts were dried over sodium sulfate and an ascorbic acid methanolic solution (15 mL, 6.58 mmol) was added, resulting in apomorphine ascorbate precipitation. The reaction mixture was stirred for additional 30 min. The salt was separated by filtration, washed with diethyl ether and dried to yield a white powder. ^1H NMR (300.1 MHz, DMSO- d_6 , δ ppm): 8.18 (d, 1H, $J = 7.9$ Hz), 7.17 (t, 1H, $J = 7.7$ Hz), 7.02 (d, 1H, 7.5 Hz), 6.69 (d, 1H, $J = 7.9$ Hz), 6.62 (d, 1H, $J = 7.9$ Hz), 4.68 (d, 1H, $J = 2.6$ Hz), 3.83 (m, 1H), 3.47 (m, 2H), 3.02 (m, 4H), 2.71 (m, 1H), 2.48 (s, 3H), 2.28 (t, 1H, $J = 14.1$ Hz). Elemental analysis for $\text{C}_{23}\text{H}_{25}\text{NO}_8$, 1.5 H_2O : calculated: C% = 58.72, H% = 6.00, N% = 2.98, H_2O %: 5.74; measured: C% = 59.00, H% = 5.75, N% = 2.82, H_2O %: 6.06.

2.3. Synthesis and characterization of 50:50 PLGA with free –COOH end groups

Benzyl alcohol (4 mmoles, 0.420 mL, 1 equiv.) was added to a blend of glycolide (60 mmoles, 6.96 g, 15 equiv.) and D,L-lactide (60 mmoles, 8.64 g, 15 equiv.) in mesitylene (30 mL). The reaction mixture was heated to 165 °C. After complete dissolution, tin octanoate (0.2 mmoles, 81 mg, 0.1 equiv. in 1 mL mesitylene) was added at once under rapid stirring. An intense boiling evolution occurred during 1 min, then the reaction mixture was kept under a moderate reflux. After completion, the reaction mixture was cooled down to room temperature and the solvent was eliminated by decantation (90% of the initial volume was recovered). The obtained polymer was dissolved in 300 mL dichloromethane and washed under stirring for 1 h with 0.5 N aqueous HCl (200 mL), then twice with water (2×150 mL). The organic solution was dried over anhydrous sodium sulfate and the solvent was eliminated under reduced pressure to yield the “protected” PLGA (with terminal benzyl ester groups) as white-off foam.

The catalyst (600 mg carbon containing 5% Pd) was added to a solution of the protected PLGA in acetone (250 mL). For complete deprotection, the reaction mixture was stirred under hydrogen atmosphere for 3 h. The catalyst was removed by filtration. The polymer was precipitated by dropwise addition (1 h) of the former solution to 2.0 L pentane under mechanical stirring. After decantation, the polymer was dissolved in dichloromethane (100 mL) and dried under reduced pressure (24 h, 50 °C, 0.5 mbar) to yield a white foam (14.1 g, 90% yield).

The number-average and weight-average molar masses (M_n and M_w) and polydispersity indexes (M_w/M_n) of the polyesters were determined by size exclusion chromatography (SEC) at 40 °C with a Waters 600 liquid chromatograph, equipped with

a Waters 2410 refractive index detector and are not corrected (eluent = tetrahydrofuran, flow rate = 1 mL/min, Waters pre-column, Waters Styragel column: HR 4E, 50–100,000 g/mol) (Waters, Guyancourt, France). Calibrations were performed using polystyrene standards (400–100,000 g/mol).

“Protected” 50:50 PLGA: ^1H NMR (300.1 MHz, CDCl_3 , δ ppm): 1.59 (m, CH_3 , 34H), 4.83 (m, CH_2 , 68H), 5.20 (m, CH, 101H), 7.37 (m, Ar, 5H). SEC: $M_n = 4810$, $M_w = 9730$, PDI = 2.02.

Deprotected 50:50 PLGA: ^1H NMR (300.1 MHz, CDCl_3 , δ ppm): 1.55 (m, CH_3 , 6H), 4.78 (m, CH_2 , 4H), 5.20 (m, CH, 2H). Here no signal was detected in the aromatic region, confirming the complete deprotection of the benzyl ester chain end. SEC: $M_n = 4620$, $M_w = 9500$, PDI = 2.05.

The corresponding data for commercial Resomer RG 502H are very similar: ^1H NMR (300.1 MHz, CDCl_3 , δ ppm): 1.59 (m, CH_3 , 3H), 4.83 (m, CH_2 , 2H), 5.20 (m, CH, 1H). SEC: $M_n = 5270$, $M_w = 10320$, PDI = 1.96.

2.4. Microparticle preparation

Apomorphine HCl-loaded, PLGA-based microparticles were prepared using (solid-in)-oil-in-water solvent extraction/evaporation techniques as follows: polymer and drug (380/360/340/320/300 and 20/40/60/80/100 mg in case of 5/10/15/20/25%, w/w, theoretical drug loading) were dissolved/dispersed within 3 mL dichloromethane by sonication (Sonopuls UW2070, 30% amplitude, 2 min; Bandelin, Berlin, Germany). If indicated, a 2.5:0.5 and 2:1 (v:v) dichloromethane:methanol or 2.5:0.5 (v:v) dichloromethane:acetone mixture was used instead of pure dichloromethane [note that in the case of the 2:1, v:v, dichloromethane:methanol mixture, the apomorphine HCl was completely dissolved in the organic phase. Thus, in this particular case, it was an oil-in-water (O/W) co-solvent extraction/evaporation technique]. This organic phase was injected using a syringe and 20 Gauge-needle into 50 mL (if not otherwise indicated) of an outer aqueous polyvinyl alcohol solution (5%, w/w, if not otherwise indicated; 4 °C; protected from light) (optionally containing 0.05, 0.1, 0.25 or 0.5 M NaCl) under stirring with a three-blade propeller (700 rpm). Stirring was continued for 20 min. The obtained particles were hardened by adding 200 mL water (4 °C) and further stirring (700 rpm, 3 min). To promote extraction of dichloromethane, the suspension was poured into 600 mL water (4 °C; protected from light) and stirred for 40 min (if not otherwise indicated) at 700 rpm. The particles were separated by filtration using a nitrocellulose membrane (3 μm), rinsed with water and subsequently dispersed in water. The suspension was frozen at -45 °C for 2 h and freeze-dried to minimize the residual solvent's content (Epsilon 2–4 LSC; Christ, Osterode, Germany) (the conditions are given in Table 1; if not otherwise mentioned “program 1” was used). Microparticles were stored under nitrogen at 4 °C and protected from light. Apomorphine ascorbate-loaded

microparticles were prepared accordingly. Optionally, a different stirring rate was applied (as indicated), or 100 mM Tris buffer pH 8.8 containing 5% (w/w) PVA was used instead of an aqueous PVA solution as outer aqueous phase (and 100 mM Tris buffer pH 8.8 was used instead of water for microparticle hardening).

2.5. Microparticle characterization

Scanning electron microscopy (SEM) and energy dispersive X-rays spectrometry (EDX) were used to study the internal and external morphology of the microparticles (S-4700 Field Emission Gun; Hitachi, Hitachi High-Technologies Europe, Krefeld, Germany; equipped with an EDX microanalysis system with a SiLi detector, Noran Instruments, Middleton, USA; Bruker's Esprit software, Madison, USA). Samples were covered under vacuum with a carbon layer. Cross-sections were obtained after inclusion into water-based glue (UHU, Buehl, Germany) and cutting with a razor blade.

Laser diffraction was used to measure mean particle diameters (Mastersizer S; Malvern, Orsay, France). The microparticles were dispersed in water.

The initial drug loading was determined as follows: approximately 10 mg microparticles were placed in 1.5 mL dichloromethane to dissolve the polymer in a glass tube. Then, 1.5 mL water (containing 0.2% ascorbic acid to avoid apomorphine oxidation) was added to extract the drug. The tube was sonicated in an ultrasonic bath for 5 min to promote apomorphine transfer from the organic into the aqueous phase. Upon centrifugation at 3000 rpm for 3 min (Centrifuge Universal 320; Hettich, Tuttlingen, Germany) the two phases were separated. This extraction procedure was conducted 3 times. The drug content in the water phase was determined by HPLC analysis (Varian ProStar 230 pump, 410 autosampler, 325 UV-vis detector, Galaxie software; Les Ulis, France). A reversed phase column C18 (Gemini 5 μm ; 110 Å; 150 mm \times 4.6 mm; Phenomenex, Le Pecq, France) was used. The mobile phase was acetonitrile:phosphate buffer pH 3 (75 mM) containing ethylenediaminetetraacetic acid (EDTA; 1 mM) (15:85, v:v), as described by Ng Ying Kin et al. (2001). The detection wavelength was 273 nm, the flow rate 1 mL/min. Fifty μL samples were injected. The elution time was around 8 min. Each experiment was conducted in triplicate.

Gas chromatography was used to determine the residual dichloromethane content of the microparticles. Samples were analyzed using a PerkinElmer Clarus 500 Gas Chromatograph, Clarus 560 Mass Spectrometer, Turbomatrix 16 HeadSpace sampler and Forte (30 m \times 0.32 mm ID) BPX volatiles column (PerkinElmer, Courtaboeuf, France). Microparticles (approximately 100 mg) were dissolved in 2 mL dimethyl sulfoxide (DMSO). Standard solutions were prepared for each solvent (in DMSO, 10 $\mu\text{L}/\text{mL}$ concentration). From these stock solutions, aliquots of 2–200 μL were added in 2 mL DMSO and used to build the calibration curves.

Table 1
Freezing-drying conditions.

Program, #	Freezing		Primary drying			Secondary drying		
	Time (h)	Temperature (°C)	Time (h)	Temperature (°C)	Pressure (mbar)	Time (h)	Temperature (°C)	Pressure (mbar)
1						10	20	
2	2	−45	10	−9	0.014	30	20	0.0014
3						10	25	
4						10	30	
5						Twice program 1		
6	Two cycles of:							
	2	−45	10	−9	0.014	30	30	0.0014

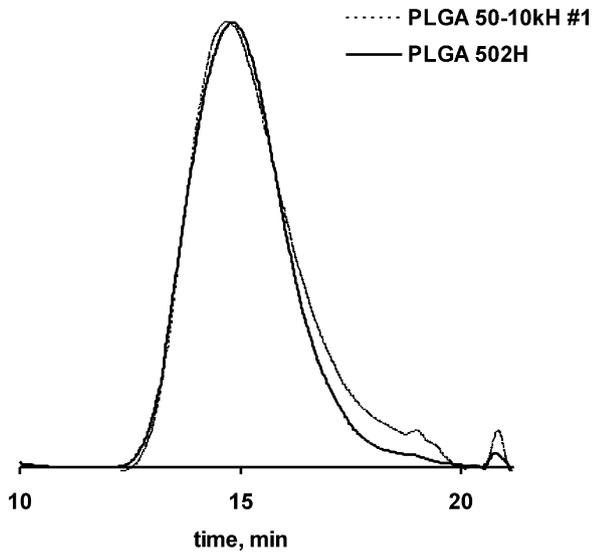


Fig. 1. SEC chromatograms of the investigated copolymers: a batch of PLGA 502H and a batch of PLGA-10kH (as indicated).

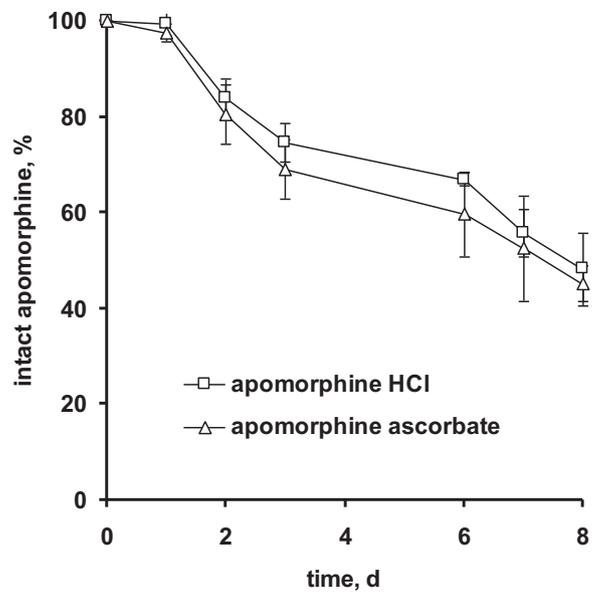


Fig. 2. Stability of apomorphine HCl and apomorphine ascorbate in phosphate buffer pH 7.4 containing 0.2% ascorbic acid at 37 °C.

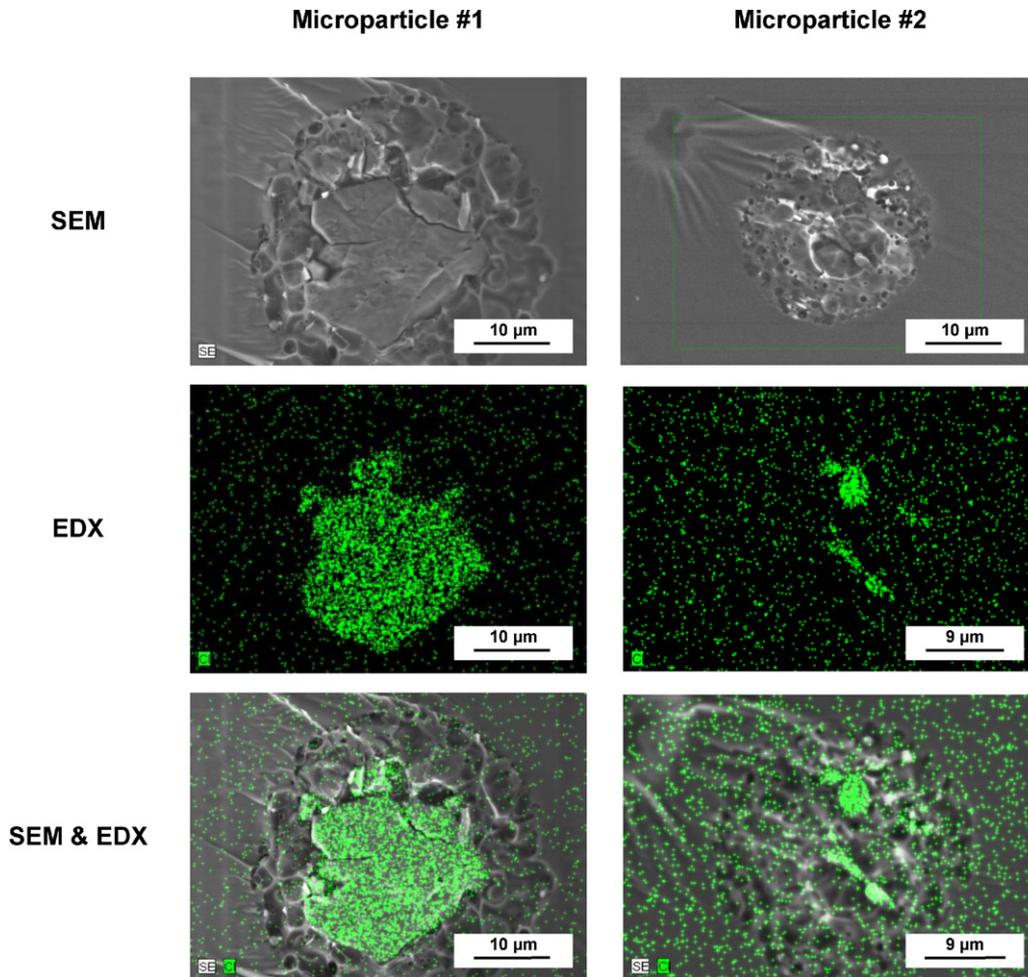


Fig. 3. Structure of two arbitrarily selected microparticles (cross-sections) loaded with apomorphine HCl and based on PLGA 502H. Upper row: SEM pictures. middle row: EDX pictures indicating the presence of chlorine (in green). Bottom row: superposition of the SEM and EDX pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.6. In vitro drug release studies

Agitated flasks: approximately 15 mg microparticles were placed in 50 mL degassed phosphate buffer pH 7.4 (USP 35) containing 0.2% ascorbic acid (to minimize apomorphine oxidation) in glass flasks. The latter were closed under nitrogen, protected from light and horizontally shaken at 37 °C (80 rpm; GFL 3033; Gesellschaft fuer Labortechnik, Burgwedel, Germany). At pre-determined time points, 3 mL samples were withdrawn (replaced with fresh medium) and analyzed by HPLC (as described above). Each experiment was conducted in triplicate. In this study, the term “burst release” refers to the amount of drug released under these conditions after 1 h exposure time.

Flow-through cells: approximately 15 mg microparticles were exposed to a continuous flow of fresh release medium in empty HPLC columns, as described in detail by Aubert-Pouessel et al. (2002). Degassed phosphate buffer pH 7.4 (USP 35) containing 0.2% ascorbic acid was pumped at 2 mL/d (syringe pump PHD 2000; Harvard Apparatus, Les Ulis, France) through HPLC columns (4.6 mm × 5 cm; Omega; Upchurch Scientific, Oak Harbor, WA, USA), placed in a water bath at 37 °C. The eluent was cooled at 4 °C and analyzed by HPLC as described above. Each experiment was conducted in triplicate.

2.7. Thermal analysis

Differential scanning calorimetry (DSC) thermograms of drug and polymer bulk powders as well as of microparticles were recorded with a DSC1 Star System (Mettler Toledo, Greifensee, Switzerland). Approximately 5 mg samples were heated in sealed aluminum pans from –10 °C to 100 °C at 10 °C/min, kept at 100 °C for 5 min, cooled at 10 °C/min to –10 °C and reheated from –10 °C to 100 °C at 10 °C/min. The glass transition temperature (T_g) was determined from the second heating cycle. To characterize the physical state of apomorphine HCl within the polymeric matrices, approximately 5 mg samples were heated in sealed aluminum pans from 0 to 300 °C at 10 °C/min.

2.8. Drug dissolution in organic solvents and aqueous solutions

To estimate the velocity and capacity of apomorphine HCl to dissolve in the inner organic phase during microparticle preparation, excess amounts of this drug were exposed to 3 mL methanol, acetone or dichloromethane in polytetrafluoroethylene (PTFE) beakers. The suspensions were sonicated for 2 min (Sonopuls UW2070, 30% amplitude) in order to provide identical conditions as during microparticle manufacturing. The supernatant was separated by filtration and analyzed for its drug content by HPLC as described above (but 10 µL organic solutions were injected in the case of methanol and acetone).

To estimate the dissolution rate of apomorphine ascorbate and apomorphine HCl in an aqueous phase, excess amounts of these salts were exposed to 1 mL phosphate buffer pH 7.4 (USP 35) containing 0.2% ascorbic acid at 37 °C in Eppendorf vials in a horizontal shaker (80 rpm; GFL 3033). The vials were closed under nitrogen and protected from light. After 1 d, samples were withdrawn, filtered and analyzed by HPLC (as described above). Each experiment was conducted in triplicate.

2.9. Drug stability in aqueous solution

The stability of apomorphine HCl and apomorphine ascorbate dissolved (20 mg/L) in degassed phosphate buffer pH 7.4 containing 0.2% ascorbic acid in glass vials at 37 °C was studied under protection from light and horizontal shaking (80 rpm). The vials were

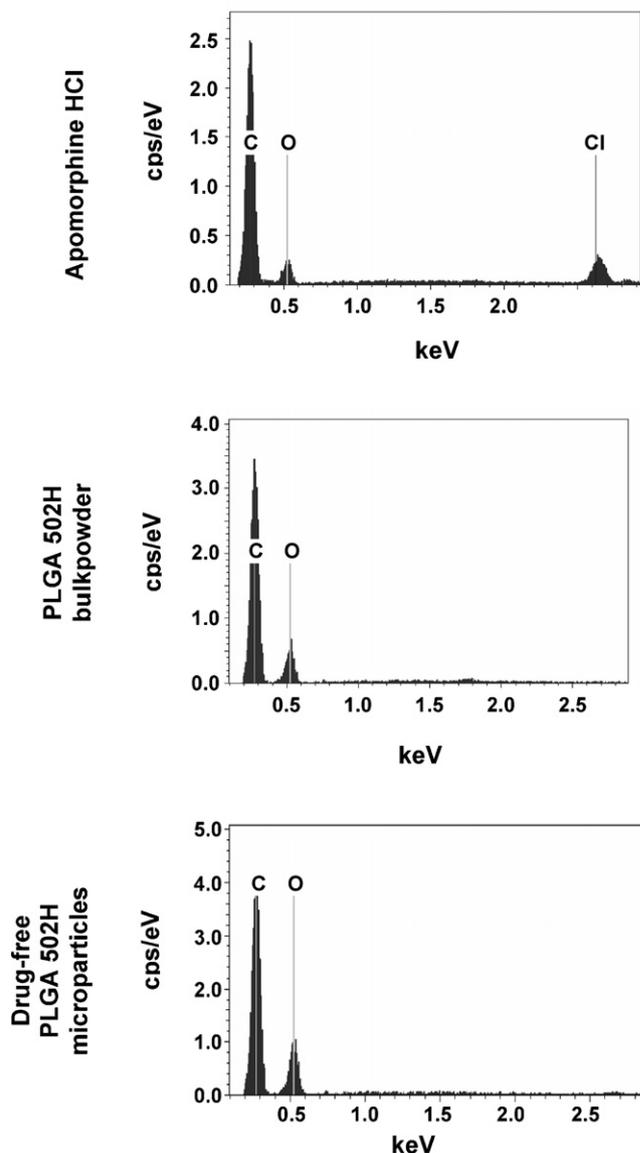


Fig. 4. EDX spectra of apomorphine HCl, PLGA 502H bulk powder and drug-free PLGA 502H-based microparticles.

closed under nitrogen and the drug contents at different time points determined by HPLC analysis as described above.

3. Results and discussion

3.1. Polymer synthesis

Two batches of 50:50 PLGA, bearing free –COOH end groups, were synthesized via ring opening polymerization. Importantly, their weight-average molar masses (M_w) and molar mass distributions (M_w/M_n) were similar to those of commercially available PLGA Resomer RG 502H: 10,340 and 9500 g/mol compared to 10,320 g/mol, and $M_w/M_n = 1.67$ and 2.05 compared to 1.96, respectively. Fig. 1 shows the molecular weight distributions of a batch of Resomer RG 502H and of a batch of synthesized polymer. Since – in addition – according to NMR data their chemical composition is identical, various key properties of these polymers are likely to be similar. To distinguish the synthesized and commercial polymers, the following terms are used in this study: “PLGA-10kH” (the

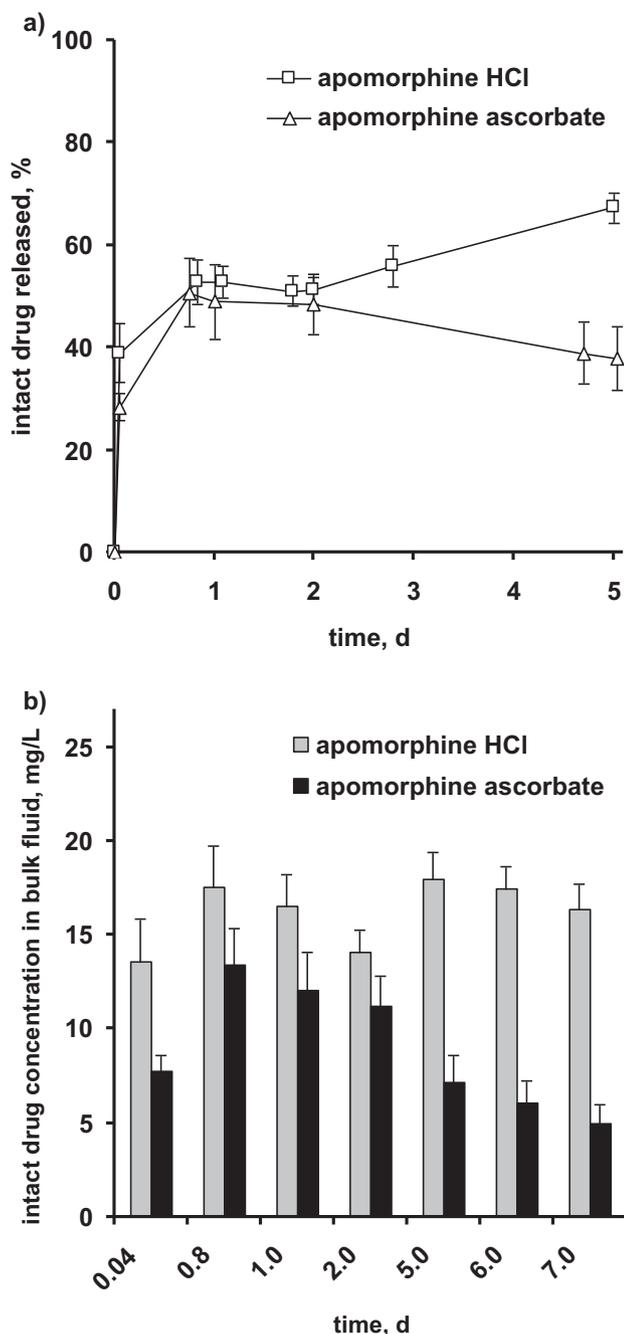


Fig. 5. Apomorphine HCl and apomorphine ascorbate release from PLGA 502H-based microparticles in phosphate buffer pH 7.4 in agitated flasks: (a) relative cumulative amounts of intact drug released (calculated based on the concentrations in the bulk fluid and withdrawn samples) and (b) experimentally measured concentrations of intact drug in the bulk fluid.

“H” indicates that free –COOH end groups are present) and “PLGA 502H”, respectively.

3.2. Apomorphine HCl versus apomorphine ascorbate

Fig. 2 shows the degradation kinetics of apomorphine HCl and apomorphine ascorbate in degassed phosphate buffer pH 7.4 (containing 0.2% ascorbic acid) at 37 °C under protection from light and horizontal shaking (80 rpm). The initial drug concentration was (20 mg/L). As it can be seen: despite the presence of the antioxidant ascorbic acid and protection from light, apomorphine was not stable: about half of the drug was degraded within 1 week.

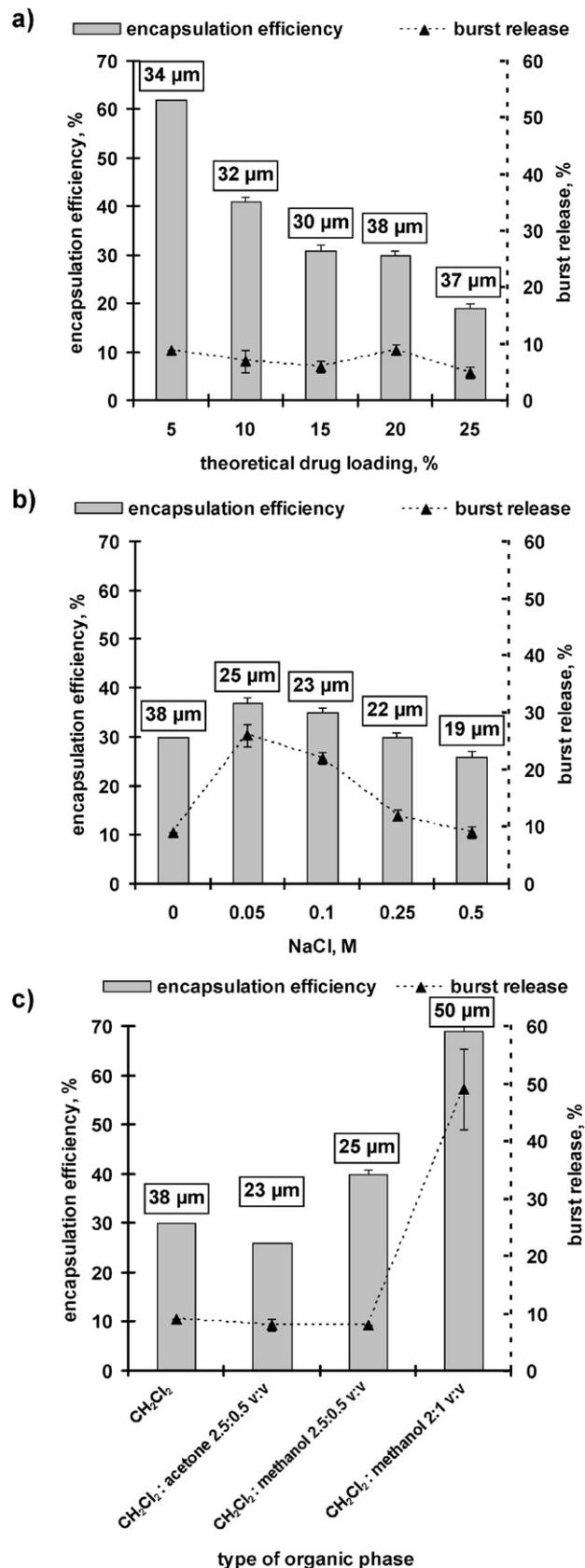


Fig. 6. Effects of the: (a) theoretical drug loading, (b) NaCl concentration in the outer aqueous phase, and (c) type of organic phase on the encapsulation efficiency, burst release (1 h) and particle size (mean diameters are indicated in the diagrams) of apomorphine HCl-loaded, PLGA-10kH-based microparticles.

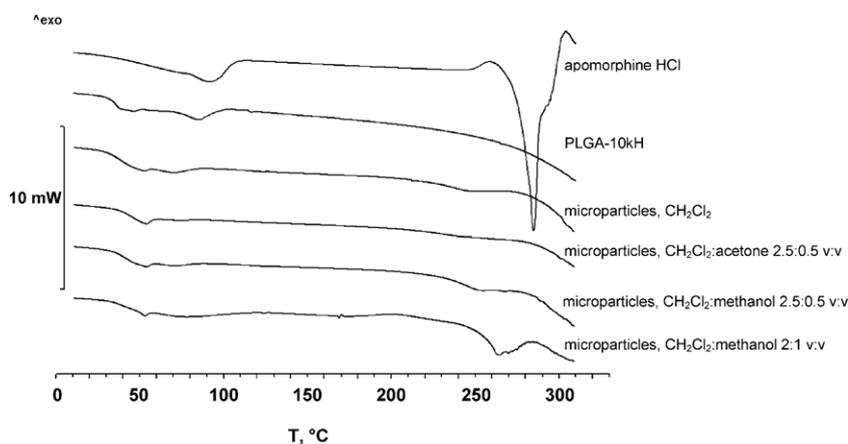


Fig. 7. DSC thermograms of apomorphine HCl and PLGA-10kH bulk powders as well as of apomorphine HCl-loaded, PLGA-10kH-based microparticles prepared with different types of organic phases (as indicated).

Importantly, the type of counter anion “chloride or ascorbate” did not significantly affect drug stability under these conditions (probably due to the relatively low ascorbate ion concentrations resulting from salt dissolution under these conditions).

Using a solid-in-oil-in-water (S/O/W) solvent extraction/evaporation technique both apomorphine salts could successfully be encapsulated within PLGA 502H-based microparticles. The yields were 80 ± 2 and $75 \pm 1\%$ for the hydrochloride and ascorbate, respectively. The particles were more or less spherical. Apomorphine ascorbate-loaded microparticles were larger than apomorphine HCl-loaded systems: 42 (Span 1.1) μm versus 33 (Span 1.4) μm . The upper row of Fig. 3 shows examples of cross-sections of apomorphine HCl-loaded particles observed by scanning electron microscopy, indicating that drug crystals of various sizes are entrapped within the polymeric matrices. Since these SEM pictures do not allow reliably distinguishing between polymer and drug, energy dispersive X-rays spectrometry (EDX) was applied. Importantly, the EDX spectrum of apomorphine HCl shows a clearly visible chlorine peak at 2.62 keV, which is absent in the spectra of the polymer bulk powder and drug-free microparticles (Fig. 4). Thus, the visualization of this 2.62 keV peak on EDX images (“mapping”) allows identifying the location of apomorphine HCl. The middle row of Fig. 3 shows such EDX mappings (in which the presence of chlorine is indicated in green). The bottom row of Fig. 3 shows the superposition of the SEM pictures of the top row and the EDX pictures of the middle row. Clearly, one large apomorphine HCl particle can be distinguished in microparticle #1 (on the left hand side). In microparticle #2 (right hand side), several smaller apomorphine HCl particles are visible.

At a theoretical drug loading of 20%, the encapsulation efficiencies for apomorphine HCl and ascorbate were $54 \pm 1\%$ (practical loading = $10.9 \pm 0.2\%$) and $42 \pm 3\%$ (practical loading = $8.5 \pm 0.6\%$), respectively. This difference might at least partially be attributed to the different dissolution rates of the two salts in aqueous solutions (e.g. 1.5 ± 0.0 and 14.6 ± 0.7 mg/mL apomorphine HCl and ascorbate were dissolved in phosphate buffer pH 7.4 at 37°C after 1 d exposure of excess amounts in Eppendorf vials under continuous stirring). Higher dissolution rates in the external phase can be expected to result in increased drug loss during microparticle preparation.

The cumulative in vitro release of intact apomorphine from PLGA 502H-based microparticles containing apomorphine HCl or apomorphine ascorbate in phosphate buffer pH 7.4 (containing 0.2% ascorbic acid) at 37°C in agitated flasks is illustrated in Fig. 5a. As it can be seen, the initial release rate (during the first 2 d) is

similar. Afterwards the cumulative amount of intact apomorphine HCl released continues rising, whereas the cumulative relative amount of intact drug released in the case of apomorphine ascorbate artificially decreases: this can at least partially be attributed to drug degradation in the release medium (Fig. 2) (despite the protection from light and presence of a nitrogen atmosphere). The cumulative amounts of drug released illustrated in Fig. 5a are calculated as the sums of the drug present in the release medium at the respective time points plus the amounts of drug present in the samples withdrawn prior to these time points. Since drug degradation in the release medium is of major importance, the “cumulative” amount of drug released seems to decrease. To avoid misleading conclusions from this type of presentation, the in vitro drug release kinetics are also plotted in a different way (Fig. 5b): the experimentally measured concentrations of intact apomorphine in the release medium are shown as a function of time. Clearly, the drug concentration significantly decreases with time after the first day in the case of apomorphine ascorbate (for the above discussed reasons). In contrast, the concentration of intact drug remains about constant in the case of apomorphine HCl (corresponding to the rising curve in Fig. 5a). This indicates that the rate at which “new” intact apomorphine is released from these microparticles compensates drug degradation in the bulk fluid (Fig. 2). Note that at the end of the observation period no intact drug remained within the microparticles, irrespective of the type of salt.

3.3. Impact of formulation and processing parameters

In this study, the term “burst release” is defined as the relative amount of intact apomorphine released into degassed phosphate buffer pH 7.4 (containing 0.2% ascorbic acid) at 37°C under protection from light and horizontal shaking (80 rpm) in agitated flasks after 1 h. During this time period, drug degradation in the bulk fluid can be considered to be very limited (Fig. 2). Apomorphine HCl-loaded microparticles based on PLGA-10kH were prepared by a (S/O/W) solvent extraction/evaporation technique. Different processing and formulation parameters were varied to evaluate their impact on the key properties of the microparticles, in particular their burst release, encapsulation efficiency and particle size.

Fig. 6a shows that increasing the theoretical drug loading from 5 to 25% (while keeping the total amount of “polymer plus drug” used during microparticle preparation constant) only moderately affected the resulting burst release and particle size, but led to steeply decreasing encapsulation efficiencies. The latter effect might at least partially be attributable to the decreasing amounts

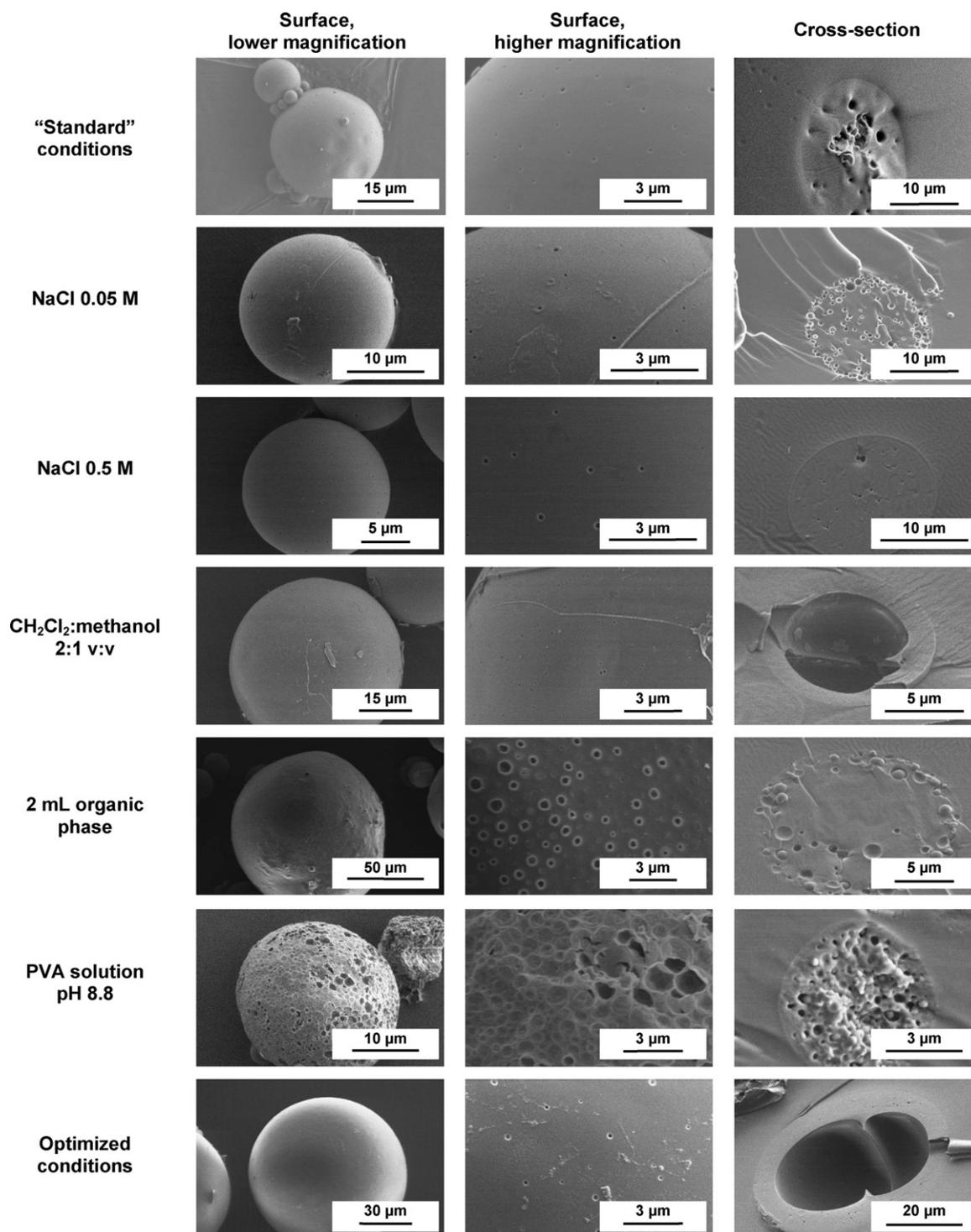


Fig. 8. SEM pictures of surfaces (lower and higher magnification) and cross-sections of the investigated apomorphine HCl-loaded, PLGA-10kH-based microparticles. In the left column the particular features are highlighted (explained in the text). “Standard conditions” means: 20% theoretical drug loading, no NaCl addition to the outer aqueous phase, 3 mL dichloromethane as internal organic phase, 50 mL 5% PVA aqueous solution pH 5.5 as outer phase, 700 rpm stirring. “Optimized conditions” means: 5% theoretical drug loading, no NaCl addition to the outer aqueous phase, 3 mL dichloromethane:methanol 2.5:0.5 (v:v) as internal organic phase, 50 mL 5% PVA aqueous solution pH 5.5 as outer phase, 400 rpm stirring.

of polymer present during microparticle formation (increasing the probability of drug crystals coming into direct contact/being lost with/into the outer aqueous phase). The fact that the initial burst was almost unaffected by the theoretical drug loading can probably be attributed to the relatively low practical drug loadings (ranging between 3 and 6%) [note that the difference in practical drug loading at 20% theoretical drug loading to the value of 10.9% indicated in

Section 3.2 can at least partially be explained by the fact that once the polymer was purchased (RG 502H) and once self-synthesized (PLGA-10kH), as described in more detail in Regnier-Delplace et al. (in press)]. In the literature often increasing drug release rates are reported when increasing the drug loading (Choi et al., 2002). The latter phenomenon might be caused by increased matrix porosity upon drug exhaust at higher drug loadings. Since in the present

study, the practical drug loadings are relatively low, this effect is not of major importance. DSC thermograms (not shown) revealed that also the glass transition temperature (T_g) was not significantly affected by the investigated variation of the theoretical drug loading, ranging between 43 and 45 °C. These T_g values are similar to the one of the polymer bulk powder (38 °C, Fig. 7), indicating that most parts of the drug are likely to be encapsulated as crystals within the polymer matrices, and not as single molecules/ions (“dissolved”) in the PLGA (in the latter case, drug–polymer interactions and shifted T_g s are likely).

The addition of 0.05 NaCl to the outer aqueous phase during microparticle preparation led to a clear increase in burst release (Fig. 6b). This might at least partially be attributed to the decrease in particle size: from 38 to 25 μm . The encapsulation efficiency increased from 30 to 37%. However, when further increasing the NaCl concentration of the aqueous PVA solution, the burst release and encapsulation efficiency decreased again, while the particle size continued decreasing. The decreased burst release might at least partially be attributable to the less porous inner microparticle structure (Fig. 8). This is consistent with reports in the literature (Luan et al., 2006) and is likely to be explained by a reduced solubility of the organic solvent in the outer aqueous phase, resulting in less rapid dichloromethane extraction and, thus, less rapid polymer precipitation.

The impact of the type of organic solvent is illustrated in Fig. 6c: the addition of 20% acetone or methanol did not significantly affect the resulting burst release, but decreased the particle size and – in the case of methanol – increased the encapsulation efficiency. The higher encapsulation efficiency in the case of methanol is probably due to the higher solubility of apomorphine HCl in methanol compared to acetone and dichloromethane: Upon sonification of a freshly prepared drug suspension for 2 min, 72.2 ± 0.9 , 0.1 ± 0.0 and 0.3 ± 0.0 mg/mL apomorphine HCl were dissolved in methanol, dichloromethane and acetone, respectively. This is consistent with the even higher encapsulation efficiency (about 70%) when further increasing the methanol content (Fig. 6c). However, in the latter case the burst release also steeply increased (to about 50%). This can be explained by the very high porosity of the microparticles obtained with dichloromethane:methanol 2:1 (v:v) blends: as it can be seen in Fig. 8, hollow spheres were obtained in this case. Due to the increased hydrophilicity of the inner phase, water more rapidly penetrates into the organic phase, leading to accelerated polymer precipitation. This is also consistent with the steep increase in microparticle size (Fig. 6c). DSC thermograms of the different microparticles (and for reasons of comparison also of the bulk drug and polymer powders) are shown in Fig. 7. In the case of apomorphine HCl, two endothermic events were observed, which can be attributed to desolvation (at 10–100 °C) and drug fusion (at about 280 °C), followed by decomposition. PLGA-10kH showed a glass transition at about 40 °C (similar to PLGA 502H). The thermograms of microparticles prepared with the different organic phases had similar T_g s (for the reasons discussed above) and partially showed drug melting peaks, especially in the case of dichloromethane:methanol 2:1 (v:v) blends. This is due to the higher practical loading of these systems (14%) and further confirms the presence of encapsulated drug crystals. The other microparticles contained less apomorphine HCl (5–8%), being closer to or eventually below the detection limit under these conditions.

Fig. 9a shows the impact of decreasing the volume of the internal organic phase from 3 to 2 mL. As it can be seen, the encapsulation efficiency remained about unaltered, whereas the particle size significantly increased (from 38 to 57 μm). This can at least partially be attributed to the higher viscosity of the inner organic phase, leading to larger droplets at the same shear forces (Sansdrap and Moës, 1993). Furthermore, the burst release significantly increased,

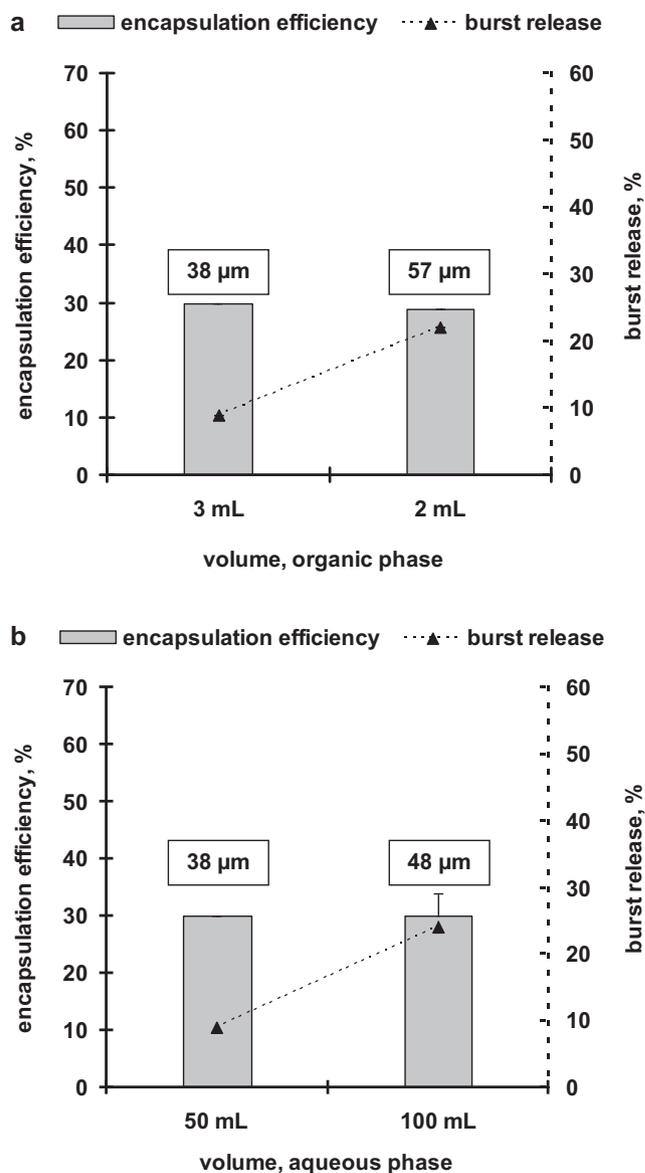


Fig. 9. Effects of the: (a) volume of the inner organic phase, and (b) volume of the outer aqueous phase on the encapsulation efficiency, burst release (1 h) and particle size (mean diameters are indicated in the diagrams) of apomorphine HCl-loaded, PLGA-10kH-based microparticles.

which can be attributed to a much more porous surface of the microparticles, as evidenced by SEM (Fig. 8).

When increasing the volume of the outer aqueous phase from 50 to 100 mL, the particle size increased as well, due to the reduced shear forces acting on the inner organic phase in the larger total fluid volume. The encapsulation efficiency remained about constant, whereas the burst release increased, probably due to the higher dichloromethane extraction rate into outer aqueous phase (higher absolute capacity to “host” organic solvent molecules), resulting in accelerated polymer precipitation and increased microparticle porosity.

When adjusting the pH of the outer aqueous phase to pH 8.8, the aqueous solubility of apomorphine can be expected to decrease due to electric charge neutrality (Subramony, 2006) [$pK_a = 7.6$, 8.8 at 25 °C; Armstrong and Barlow, 1976]. As it can be seen in Fig. 10a, the encapsulation efficiency almost doubled. However, the burst release tremendously increased (to about 50%), due to the very high internal and external porosity of the microparticles (Fig. 8) and

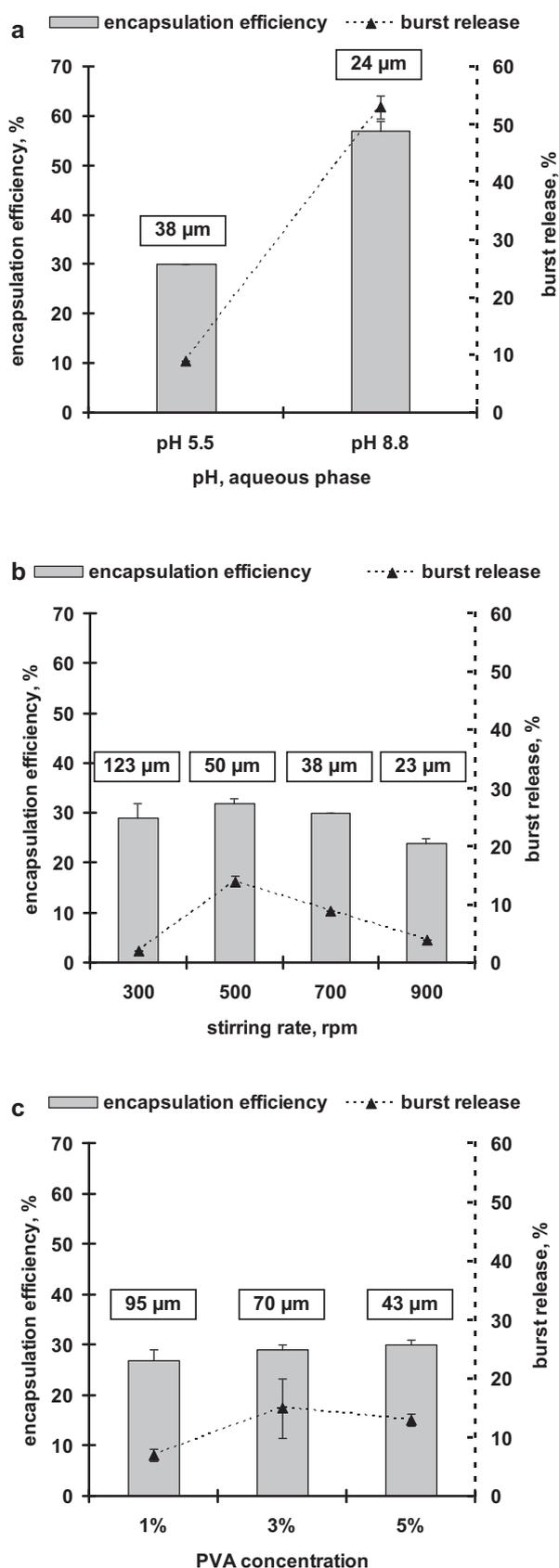


Fig. 10. Effects of the (a) pH of the outer aqueous phase, (b) stirring rate, and (c) polyvinyl alcohol (PVA) concentration in the outer aqueous phase on the encapsulation efficiency, burst release (1 h) and particle size (mean diameters are indicated in the diagrams) of apomorphine HCl-loaded, PLGA-10kH-based microparticles.

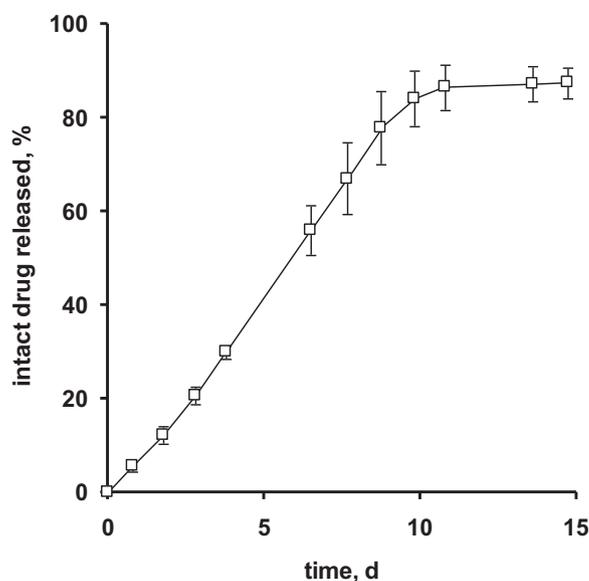


Fig. 11. Cumulative release of intact apomorphine HCl from PLGA-10kH-based microparticles in phosphate buffer pH 7.4 in flow-through cells (2 mL/d) prepared by an O/W co-solvent method (dichloromethane:methanol 2.5:0.5 (v:v), 5% theoretical drug loading, 50 mL of 5% polyvinyl alcohol solution, 400 rpm stirring).

the reduced particle size (Fig. 10a). In addition, the microparticles became green, indicating drug oxidation (being accelerated at elevated pH, Burkman, 1965).

As expected, the microparticle size decreased when increasing the stirring rate from 300 to 900 rpm (due to the increased shear forces, resulting in smaller organic phase droplets) (Fig. 10b). The encapsulation efficiency was almost unaffected, whereas the burst release first increased and then decreased again.

Increasing the PVA concentration in the outer aqueous phase led to a decrease in particle size from 95 to 43 μm , mainly due to the surfactant properties of PVA (facilitating the creation of smaller organic phase droplets). As it can be seen in Fig. 10c, the encapsulation efficiency remained about unaltered, whereas the burst release increased with increasing PVA concentration (at least partially due to the decrease in particle size).

3.4. Microparticles with zero order release kinetics

Based on the obtained results, the following process and formulation parameters were selected to provide maximized encapsulation efficiency and limited burst release (in the following called "optimized conditions"): 5% theoretical drug loading, 3 mL dichloromethane:methanol mixture (2.5:0.5, v:v) (thus, this is in fact an O/W extraction/evaporation method), 50 mL 5% PVA aqueous solution pH 5.5 (free of NaCl), and 400 rpm (to target a particle size between 50 and 100 μm) stirring rate. The resulting yield was 90%, the practical drug loading $4.1 \pm 0.1\%$ (corresponding to $81 \pm 3\%$ encapsulation efficiency). The mean diameter of the particles was 83 μm , the glass transition temperature 40 $^{\circ}\text{C}$ (determined by DSC analysis). SEM pictures showed that hollow spheres were obtained with a smooth, slightly porous surface (Fig. 8). In vitro drug release in degassed phosphate buffer pH 7.4 (containing 0.2% ascorbic acid) at 37 $^{\circ}\text{C}$ in flow through cells (2 mL/d, empty HPLC columns) is shown in Fig. 11. This type of set-up reduces the above discussed artificial drug degradation in the release medium (Delplace et al., 2012). Clearly, about zero-order drug release kinetics (constant drug release rate) was observed during 10 d. About 90% intact apomorphine HCl was released at that stage, the complementary 10% were likely degraded within the microparticles and/or in the bulk fluid (flow through cells allow reducing artificial drug degradation,

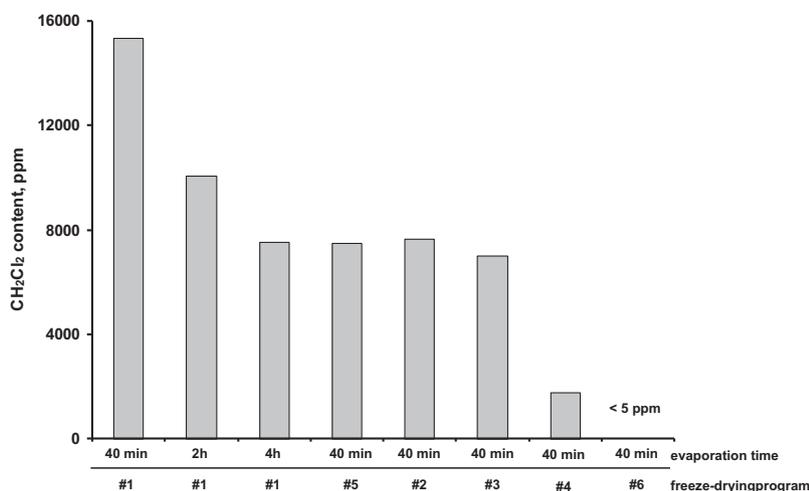


Fig. 12. Residual organic solvent's content (dichloromethane) of apomorphine HCl-loaded, PLGA-10kH-based microparticles as a function of the evaporation procedure and freeze-drying conditions (Table 1).

but do not necessarily completely suppress it). The fact that the release rate was constant might at least partially be attributed to the particular geometry of these microspheres (being hollow): in contrast to “filled” spheres the increase in the length of the diffusion pathways with time is less pronounced. In purely diffusion controlled drug delivery systems, often monotonically decreasing release rates are observed, because the distance to be overcome by the drug increases with time: first surface-near drug is released, later drug located at the center. In these hollow microspheres this increase in the length of the diffusion pathways is limited and seems to be compensated by the increase in drug mobility within the polymeric system (due to PLGA degradation).

3.5. Residual dichloromethane content

Fig. 12 shows the dichloromethane contents of microparticles prepared under “standard conditions” (20% theoretical drug loading, no NaCl addition to the outer aqueous phase, 3 mL dichloromethane as internal organic phase, 50 mL 5% PVA aqueous solution pH 5.5 as outer phase, 700 rpm stirring). The freeze-drying conditions were varied as indicated in Table 1. In addition, the time to allow for dichloromethane evaporation during microparticle preparation (prior to filtration) was varied from 40 min to 4 h. As it can be seen in Fig. 12, the increase in evaporation time led to decreased residual solvent contents. However, the microparticles became green, indicating the oxidation of surface-near apomorphine. A repetition of the same freeze-drying program (#5, 40 min “evaporation time”) resulted in a similar reduction as 4 h evaporation prior to filtration, but without color change. The same was true when increasing the secondary drying time during freeze-drying from 10 to 30 h (#2), or when increasing the secondary drying temperature from 20 to 25 °C (#3). Interestingly, the residual dichloromethane content was significantly reduced when increasing the latter temperature to 30 °C (#4). Importantly, a further substantial reduction was obtained when increasing the secondary drying time to 30 h and running two cycles (#6). In this case the dichloromethane content fell below the detection limit (under the given experimental conditions = 5 ppm), thus, well below the currently authorized limit of 600 ppm.

4. Conclusions

The presented apomorphine HCl-loaded, PLGA-based microparticles can be expected to offer an interesting potential for

improved rescue treatment of patients suffering from advanced Parkinson's disease, experiencing end-of-dose and/or unpredictable “off” phases. The labile drug is effectively protected by the biodegradable and biocompatible polymer and its release rate is well controlled during about 10 d.

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