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Preparation and Characterization of Poly(D,L-Lactide) (PLA) and Poly(D,L-Lactide)-Poly(Ethylene Glycol) (PLA-PEG) Nanocapsules Containing Antitumoral Agent Methotrexate

Tatiany J. de Faria, Angela Machado de Campos, Elenara Lemos Senna*

Summary: The aims of the present work were to prepare and characterize nanocapsules containing antitumoral agent methotrexate (MTX) from poly(D,L-lactide) (PLA) and poly(D,L-lactide)-poly(ethylene glycol) diblock copolymer (PLA-PEG) with the purpose of administrating this drug by topical ocular route for primary ocular lymphoma treatment. Nanocapsules were prepared by the interfacial deposition of preformed polymer. The influences of the initial amount of MTX on the encapsulation efficiency, drug recovery and drug content, as well as the physicochemical properties of the particles were evaluated. The particle mean diameters were 246 and 146 nm, and zeta potential values were −38.8 and −33.6 mV, for the MTX-loaded nanocapsules prepared from PLA and PLA-PEG, respectively. The methotrexate content in the particles increased with the increasing in the drug amount added to the formulations, but the drug recovery decreased significantly. After 4 h of *in vitro* release, 28 and 86% of MTX was released from PLA and PLA-PEG nanocapsules, respectively.

Keywords: diblock copolymers; drug delivery systems; methotrexate; poly(D,L-lactide); poly(D,L-lactide)-poly(ethylene glycol)

Introduction

Over the past decades, there has been considerable interest in developing biodegradable nanocapsules as effective drug delivery systems. Nanocapsules are submicronic nanoparticulate carriers often composed of an oil core surrounded by a polymeric wall with lipophilic and/or hydrophilic surfactants on the interface. These colloidal carriers have shown the following advantages: (i) drug protection against *in vivo* degradation; (ii) stability; and (iii) ability to control drug release. Nanocapsules have been considered unique among colloidal carrier systems. They possess the vesicular organization of liposomes, but with an internal oil reservoir, allowing the encapsulation of liposoluble drugs, and a polymeric network surrounding the oily core which renders them more stable than nanoemulsions.[1,2]

In nanocapsule formulation, particular interest has been focused on the use of polyesters materials such as poly(D,L-lactide) (PLA). The great advantage of this polymer is its biodegradability by simple hydrolysis of the ester backbone in aqueous environments such as body fluids. Furthermore, the degradation products are ultimately metabolized to carbon dioxide and water, or are excreted via kidney.[3]

Several techniques have been used to prepare nanocapsule suspensions. The interfacial deposition of preformed polymers is the simplest and most advantageous method. The procedure consists in mixing a water-miscible organic phase containing oil

Laboratório de Farmacotécnica, Programa de Pós-Graduação em Farmácia, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Florianópolis, CEP 88040-900, Brasil
E-mail: tatyfaria@ccs.ufsc.br

(with or without lipophilic surfactant) with an aqueous phase containing a hydrophilic surfactant. The formation of nanocapsules was explained by interfacial turbulence generated during the fast diffusion of the water-miscible solvent in water which, in turn, provides energy for oil droplet formation. Once solvent diffusion is complete, the polymer aggregates around the oil droplets. Using this technique, monodispersed nanoparticles can be prepared easily in one step without further purification and with a very high yield of encapsulation for lipophilic substances.[2,4]

Methotrexate (4-amino-10-methylfolic acid) (MTX) is a drug widely used for the treatment of various neoplasic diseases in adults, mainly for the treatment of the primary ocular lymphoma, a subset of the primary central nervous system lymphoma where malignant lymphoid cells invade the retina, vitreous body, or optic nerve head.[5] Topical application of this drug into to the eye might be considered advantageous to the treatment of ocular lymphoma, since it may avoid systemic undesirable effects of the drug. Unfortunately, due to the rapid and extensive precorneal loss caused by drainage and high tear fluid turnover, only a small fraction of the applied drug penetrates the cornea and reaches intraocular tissues.[6] Thus, the current management of ocular lymphoma included a topical therapy based on intravitreal injections of the drug. However, intravitreal injections present severe risks of systemic and local toxicity and may lead to glaucoma and hemorrhage.[7]

On the other hand, several studies have shown that nanoparticles are efficient to improve the precorneal residence time and/or penetration ability of the active agent, prolonging the duration of the action and increasing bioavailability in ocular tissues.[6,8] Furthermore, the coating of the colloidal carriers with specific polymers such as polyethylene glycol has demonstrated to be useful in improving the interaction of particles with the corneal epithelium and the nanoparticle stability in the tear.[8] Nanocapsules containing hydrophilic polyethylene glycol chains on the surface can be prepared using a new class of PLA-PEG diblock copolymers obtained by a ring-opening polymerization method. In this work, we have investigated the MTX loading capacity and physicochemical characteristics of PLA and PLA-PEG nanocapsules intended to ocular administration. The release profiles of the drug from PLA and PLA-PEG nanocapsules have also been evaluated and compared.

Materials and Methods

Materials

Poly(D,L-lactide) was supplied from Boehringer Ingelheim (France). Poly(D,L-lactide)-poly(ethylene glycol) diblock copolymer (PLA-PEG 49 kD, 20% PEG 5 kD) was obtained from Alkermes (EUA). MTX was obtained from Pharmacia (Miantrex®, Australia). Miglyol 812 N and sorbitan monooleate (Span 80) were purchased from Sasol (EUA) and Beraca (Brazil) respectively. Polysorbate 80 (Tween 80) was obtained from Delaware (Brazil). All other chemicals were of analytical grade, except those used for HPLC analysis.

Methods

Preparation of Colloidal Nanocapsule Suspensions

Suspensions of nanocapsules of PLA and PLA-PEG containing methotrexate were prepared by the interfacial deposition process described by Fessi et al.[9] Briefly, 40 mg of polymer (PLA or PLA-PEG) was dissolved in 10 ml of acetone containing 125 μl of Miglyol 812 N, 40 mg of Span 80 and different amounts of MTX (1.25, 2.50, 5.0, 7.5 or 10.0 mg). This organic solution was poured into 20 ml of aqueous phase containing 0.15% (w/v) of Tween 80 and pH 5.0 adjusted with percloric acid under moderate magnetic stirring. The organic solvent was evaporated under reduced pressure and the final volume was adjusted to 10 ml. The final nanocapsule suspensions

were then filtered through 0.8 μm cellulose ester membranes.

Determination of the Encapsulation Efficiency (EE), Drug Content and Drug Recovery

An aliquot of each MTX-loaded nanocapsule batch was dissolved in acetonitrile and total methotrexate content was then assayed by reversed-phase HPLC using the following conditions: column, Supelcosil LC-18 (150 × 4.6 mm ID, 5 μm, Supelco, EUA); mobile phase, water:acetonitrile: tetrahidrofurane (45:50:5 v/v) adjusted to pH 3.0 with percloric acid; flow rate, 1.0 $ml \cdot min^{-1}$ and UV detection at 313 nm. The aqueous phase containing free MTX was separated after the ultrafiltration/centrifugation of suspensions at 10 000 rpm for 20 min in Ultrafree-MC 100000 devices (Millipore, EUA). The supernatant was removed and the free drug content was determined by HPLC as described above. The samples were injected in triplicate and MTX concentration was calculated by comparing the peak area corresponding to the drug with that obtained from a MTX standard solution analyzed in the same conditions. Encapsulation efficiency (%) was calculated as being the difference between the total and the free MTX concentration in the nanocapsule suspension. The recovery (%) was estimated correlating the total amount of MTX found in the suspension, with that initially added to the formulation.

Particle Size and Zeta Potential Determination

The mean particle size and zeta potential of nanocapsules were determined by photon correlation spectroscopy (PCS) and laser doppler anemometry (LDA), respectively, after the dilution of suspensions with bidistilled water, using a Zetasizer 3000HS (Malvern Instruments, UK).

In vitro Drug Release Study

The *in vitro* drug release studies were performed using the diffusion technique. Two milliliters of nanocapsules or a MTX solution were placed in a dialysis bag (Spectra/Por CE MWCO 10000, EUA) and the sample-filled tube was hermetically sealed and put into 200 ml of Ringer buffer (pH 7.0) maintained at 37 °C under magnetic stirring. At appropriated time points, aliquots (3.0 ml) of medium were withdrawn and replaced with fresh buffer. The MTX concentration in the samples was determined by spectrofluorimetry method after MTX oxidation with permanganate potassium as described by Espinosa-Mansilla et al.[11] In short, aliquots of release medium (2.0 ml), 0,6 ml of pH 5.0 acetic acid/sodium acetate buffer solution ($C = 0.5$ M), 0.3 ml of 1×10^{-3} M potassium permanganate solution, 0.10 ml of 60 $\mu g \cdot ml^{-1}$ ascorbic acid solution were mixed and diluted with ultrapure water up to a final volume of 3 ml. After 200 s of reaction time, acquisition of fluorescent measurements were made on a Hitachi F4500 spectrofluorimeter, and curves were registered at $\lambda_{exc} = 350$ nm and $\lambda_{em} = 457$ nm. A calibration curve was previously constructed in the range concentration from 0.15 to 1.0 $\mu g \cdot ml^{-1}$. The methotrexate concentration was estimated comparing the emission spectra areas of samples with those obtained with a standard solution of the drug analyzed on the same conditions. The analyses were carried out in triplicate and MTX release (%) versus time (min) profiles were plotted.

Results and Discussion

The encapsulation of drugs in nanocapsules has been reported to be highly dependent on the drug solubility in both oil and aqueous phase. It may be possible that the encapsulation of lipophilic drugs in nanocapsules may reach close to 100% when formulation conditions are optimized. MTX is the prototype folate antagonist cytotoxic drug. Its molecule is made up of a heterocyclic portion (a 2,4-diamino-substituted pterine ring) linked to a aminobenzoil portion, which is, in turn, an amide bonded to a glutamic acid unit (Figure 1). Hence, MTX is a polyelectrolyte carrying two carboxyl groups—with dissociation

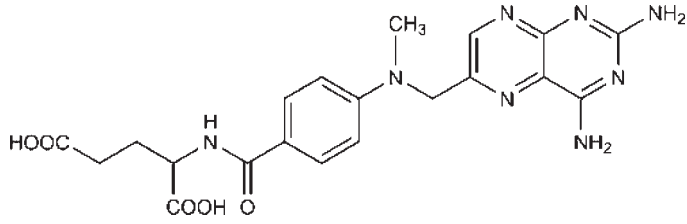

Figure 1.
Chemical structure of MTX.

constants (pKa) of 3.36 (α-carboxyl) and 4.70 (γ-carboxyl)—and a number of potentially protonated nitrogen functions, the most basic of which, presumably, the guanidinic *N*-1 on the pterine ring (pka 5.71). As a consequence, the MTX water solubility is pH-dependent, ranging from 0.9 mM at pH 5.0 to 20 mM at pH 7.[11] Therefore, aiming to assure high encapsulation values, the aqueous phase of formulation was adjusted to pH 5.0 before nanocapsule formation.

In order to establish the loading capacity of nanocapsules regarding the incorporation of MTX, a formulation study was carried out using different amounts of drug dissolved in the organic phase of the formulation. Table 1 shows the effect of drug amount and the type of polymer (PLA or PLA-PEG) on encapsulation efficiency, drug recovery, and MTX content of nanocapsule suspensions. As can be observed, for both polymers studied, higher encapsulation efficiency and drug recovery values were obtained when 1.25 and 2.50 mg of MTX were added, subsequently decreasing considerably as more MTX was added to the organic phase. Further, subsequent MTX amounts not only lead to an increase of the drug content, but they also lead the increasing of the dissolved drug in the external phase.

Particle Size and Zeta Potential Determination

Nanoparticles are characterized by their mean particle diameter and charge surface. The average diameters and zeta potential values of unloaded and MTX-loaded nanocapsules obtained from PLA and PLA-PEG are listed in Table 2. As can be observed, the physicochemical properties of the nanocapsules were affected by the coating of particles with polyethylene glycol. In fact, when surfactant is used, the nanoparticles have a negative surface charge, as indicated by the negative values of the zeta potential. This negative surface charge is related to the presence of carboxyl end groups (from PLA), located near the surface and/or to the presence of adsorbed surfactants.[12] In contrast, the presence of

Table 1.
Results of MTX content (μg), encapsulation efficiency (%) and MTX recovery obtained after determination of drug concentration by HPLC.

Formulation	Polymer	Initial amount MTX	Drug content	EE	Drug recovery
		mg	$\mu g \cdot ml^{-1}$	%	%
NC_1		1.25	88.27 ± 11.35	79.31 ± 15.85	70.62 ± 9.08
NC_2		2.50	170.01 ± 39.77	43.88 ± 13.00	77.48 ± 5.75
NC_3	PLA	5.00	230.88 ± 123.69	40.45 ± 16.08	46.17 ± 24.74
NC_4		7.50	310.08 ± 87.77	29.98 ± 15.39	41.35 ± 11.71
NC_5		10.0	280.31 ± 96.84	24.87 ± 5.78	28.03 ± 9.68
NC_6		1.25	78.91 ± 24.56	59.97 ± 14.39	63.13 ± 19.64
NC_7		2.50	161.60 ± 21.03	66.28 ± 2.52	64.64 ± 8.41
NC_8	PLA-PEG	5.00	316.42 ± 47.18	21.18 ± 5.74	53.29 ± 19.44
NC_9		7.50	233.59 ± 84.52	42.03 ± 2.34	31.14 ± 11.27
NC_{10}		10.0	397.07 ± 53.45	24.31 ± 0.29	39.71 ± 5.34

Table 2.
Zeta potential (mV) and mean diameter (nm) of unloaded and MTX-loaded nanocapsules.

Nanocapsules	Zeta potentials	Diameters[b)]
	mV	nm
PLA		
Unloaded nanocapsules	−33.2 ± 0.5	251.9 (0.17)
NC_1 (1.25 mg)[a)]	−38.8 ± 1.2	267.9 (0.10)
NC_2 (2.50 mg)[a)]	−13.4 ± 1.2	377.2 (0.14)
PLA-PEG		
Unloaded nanocapsules	−22.1 ± 1.4	169.2 (0.12)
NC_6 (1.25 mg)[a)]	−33.6 ± 1.8	146.3 (0.14)
NC_7 (2.50 mg)[a)]	−33.6 ± 0.5	161.4 (0.11)

[a)] Initial amount of MTX.
[b)] In parenthesis = polydispersion index.

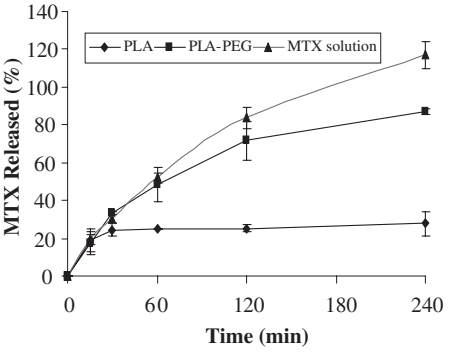

Figure 2.
MTX release profiles from PLA and PLA-PEG nanocapsules.

PEG chains on the surface of the particles increased the zeta potential from −38.8 to −33.6 mV. This effect is explained by the shifting of the shear plane of the diffuse layer to a larger distance from the particles.[12] On the other hand, the increasing of MTX content in the nanoparticle suspension led to an increase of the zeta potential for nanocapsules prepared from PLA, but not for those prepared from PLA-PEG. In addition, unloaded nanocapsules exhibit higher zeta potential values. These results may be correlated either to the location of the drug on the surface of the particle or to the drug dissolution in vehicle, hence, affecting the charge global balance of the suspensions. The use of the PLA-PEG diblock copolymer also led to the reduction of mean diameter, as it can be observed in Table 2. This effect could be attributed to the amphiphilic property of the PLA-PEG polymer and, thus, to its ability to reduce the interfacial tension during the nanocapsule formation. However, the low values of polydispersed index indicated the presence of monodispersed particle populations for all tested formulations.

In vitro Drug Release Study

MTX release studies were carried out with the nanocapsules prepared with addition of 1.25 mg of the drug. Because it rapidly separates particles from the medium, membrane diffusion is the most widely used experimental method for the evaluation of the *in vitro* release profiles of drugs incorporated in nanoparticles. In this study, fluorimetry was employed to determine the MXT concentration in the medium as a very specific, sensitive, and rapid technique. However, while some natural folates are fluorescent per se, MTX and its metabolites are not; the fluorescent derivative 2,4-diaminopteridine-6-carboxilic acid is obtained after drug oxidation with potassium permanganate.[10,13] The release profiles of MTX are displayed in Figure 2, which shows that MTX is released slower from PLA (28% in 4 h) than it is from PLA-PEG nanocapsules (86% in 4 h). A MTX solution was used as a control to verify the effect of the dialysis membrane on release rate of the drug. In this case, the complete diffusion of the drug across the membrane was verified after 4 h. As a general rule, the release of drugs from nanocapsules has been described as being governed only by the partition coefficient of the drug between the oily core and the aqueous medium, and the relative volumes of these two phases. However, the effect of the molecular weight, nature of the polymeric barrier and PEG to PLA ratio in the copolymer on drug release profiles from nanoparticles has been described in previous works.[14] Besides, in both cases, the presence of the polymeric wall delayed the MTX release from nanocapsules, as demonstrated by the rapid diffusion of the

drug through the membrane, when a MTX solution was placed into the dialysis bag.

Conclusion

PLA and PLA-PEG nanocapsules containing methotrexate were obtained with high encapsulation efficiency and drug recovery values. Both the particle size and the zeta potential were, nonetheless, affected by coating the particles with poly(ethylene glycol) and nanocapsule drug content. The drug release was affected by the nature of the polymeric barrier. In view of this, MTX-loaded PLA-PEG nanocapsules can provide new opportunities to improve current ocular MTX-based therapies, widening the scope of biomedical applications for this drug.

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