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Pentamidine-loaded poly(D,L-lactide) nanoparticles: physicochemical properties and stability work

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Abstract

This work describes the preparation, the physicochemical properties and the stability of pentamidine-loaded poly(D,L-lactide) nanoparticles. The nanoprecipitation method was used and various concentrations of phospholipids and poloxamer tested. The pentamidine concentration was 0.5 mg/ml. The percentage of pentamidine binding varied and was dependent on the phospholipid concentration. The highest binding percentage ($75.8\% \pm 1.8$) was obtained with the highest concentration of phospholipids (1.25% w/v). All formulations tested presented monodispersed nanoparticles with a mean diameter of between 131 and 154 nm. The pH of tested formulations ranged from 7.5 to 8. After 9 months of storage at 4°C, all formulations were stable. Nevertheless, a pentamidine release of between 12 and 21% was observed. Exponential dependence of the amount of drug release on time was evidenced ($r > 0.98$). The stability studies have also shown a significant decrease of pH (2 units), a nanoparticle size not significantly modified and an unchanged morphology. © 1997 Elsevier Science B.V.

Keywords: Pentamidine; Nanoparticles; Poly(D,L-lactide); Physicochemical properties; Stability; Visceral leishmaniasis

1. Introduction

Treatment with drug loaded carriers has been suggested as a method of improving the chemotherapy currently in use for visceral leishmaniasis (Alving et al., 1986). Colloid carriers are

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preferentially taken up by cells of the mononuclear phagocyte system (MPS) and thus target the drug directly to the parasitized host cells (Youssef et al., 1988; Puisieux et al., 1994). To improve the efficacy of antileishmanial drugs, different carriers, mainly represented by liposomes, microparticles and nanoparticles, were used. Most of the studies concerned liposomes.

Pentavalent antimonials, the first line drugs currently used for the treatment of visceral leishmaniasis (Modabber et al., 1992), were the first compounds tested with liposomes (Alving et al., 1978). However, the commercial development of liposomal stibogluconate was abandoned because of its toxicity in monkeys (New and Chance, 1980). Many other drugs carriers have been studied in experimental visceral leishmaniasis (Croft et al., 1989). Among them, only liposomal amphotericin B was used in clinical studies (Davidson et al., 1991, 1996; Torre-Cisneros et al., 1993). Nevertheless, the problem of stability of phospholipid vesicles during the storage and in biological fluids limited their use.

Nanoparticles could represent an interesting alternative to liposomes. Biodegradable carriers as poly(D,L-lactide) and polyisohexylcyanoacrylate were used. Amphothericin B was incorporated into poly(D,L-lactide-coglycolide) nanoparticles (Venier-Julienne et al., 1995) but was only tested in vitro. Primaquine, a minor antileishmanial drug, was loaded on polyisohexylcyanoacrylate (PIHCA) and on poly(D,L-lactide) polymers (Gaspar et al., 1991; Rodrigues et al., 1995). Polyhexylcyanoacrylate carriers appeared to be toxic in mice (Gaspar et al., 1992). Primaquine associated with poly(D,L-lactide) nanoparticles was also tested in a *Leishmania donovani* infected mice model showing an higher activity than free drug (Rodrigues et al., 1994).

At last, pentamidine, a major antileishmanial drug which has long been a second line treatment of leishmaniases after antimony failure or intolerance (Davidson and Croft, 1993), was loaded on several carriers. The first studies demonstrated that the encapsulation of pentamidine in human red cell ghosts allowed to increase significantly its efficacy in a visceral leishmaniasis hamster model (Berman et al., 1986). The encapsulation of pentamidine in liposomes was performed to improve the tolerance, reduce the side effects and enhance the uptake in the lungs in pneumocystosis treatment (Debs et al., 1987).

We have previously loaded pentamidine on polymethacrylate nanoparticles and studied the in vitro delivery of the drug from this carrier (Paul et al., 1997). We have also performed in vitro and in vivo experiments to evaluate the action of pentamidine-bound nanoparticles against intracellular *Leishmania* (Deniau et al., 1993; Fusaï et al., 1994). Pentamidine loaded on polymethacrylate nanoparticles was 6 fold more active than free drug in *Leishmania infantum* infected BALB/c mice (Durand et al., 1997). These studies showed that targeted pentamidine should be of major interest in the treatment of visceral leishmaniasis. The main problem of these nanoparticles was their low biodegradability (Rolland, 1987). This poor biodegradability could be an inconvenient to their in vivo administration. Conversely, polyester polymers are very interesting for substained drug delivery as they are known to be completely biodegradable and well tolerated by tissues (Kulkarni et al., 1971). Investigations on the potential use of poly(L-lactic acid) and poly(D,L-lactic acid) for medical purposes were first reported by Kulkarni et al. (1966). Polyesters are slowly hydrolysed into lactic acid, a metabolite of Krebs cycle (Bazile et al., 1992). This polymer is degraded via non enzymatic hydrolysis in aqueous solution in a temperature- and pH- dependent fashion (Makino et al., 1985). Therefore, the chemical stability of any poly(D,L-lactide)-based drug delivery system must be evaluated (Magenheim and Benita, 1991). At last, these polymers have already demonstrated their interest in visceral leishmaniasis with primaquine (Rodrigues et al., 1994 and Rodrigues et al., 1995).

This paper describes the preparation of pentamidine-loaded poly(D,L-lactide) nanoparticles and focuses on their physicochemical properties and stability.

Table 1
Composition of different formulations (mg)

Formulation	A	B	C	D	E	F
Pentamidine base	5	5	5	5	5	5
Poly(D,L-lactide) polymer	125	125	125	125	125	125
Poloxamer	300	250	250	250	250	125
Soybean lecithin (PL)	60	60	75	100	125	125
Poloxamer/PL ratios	5	4.17	3.33	2.5	2	1

2. Materials and methods

2.1. Materials

Pentamidine isethionate was purchased from Bellon (Neuilly sur Seine, France). The phospholipid mixture (PL) (Lipoid S 75, soybean lecithine with a level of purity of 70%) was kindly supplied by Lipoid GmbH (Ludwigshafen, Germany). Poloxamer 188 (Symperonic PE/F-68®) was purchased from ICI (Clamart, France). Polymer poly(D,L-lactide) (PLA, mol. wt. (GPC) 200 000) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Other reagents were analytical grade.

2.2. Production of free base form of pentamidine

Pentamidine base was previously obtained by precipitation of pentamidine isethionate solution in alkaline medium (25% ammonium hydroxide) at 4°C under magnetic stirring. The precipitate was filtered, washed twice with cold ammoniacal water and dried under vacuum. Pentamidine base obtained was characterized by UV (Jasco 7800, Prolabo, Paris, France) and infrared spectroscopy (Perkin Elmer 2000 FT-IR, St Quentin en Yvelines, France).

2.3. Preparation of PLA nanoparticles

PLA nanoparticles were prepared according to the method reported by Fessi (Fessi et al., 1992). Pentamidine base, phospholipids and PLA were dissolved in acetone (25 ml). This solution was mixed with the alkaline aqueous solution (1 ml of 25% ammonium hydroxide) containing Poloxamer 188. After 15 min of stirring, the acetone and part of the water were evaporated on a rotary evaporator under reduced pressure (0.8 bar) at 60°C. The final volume was 10 ml. Different concentrations of phospholipids ranging from 0.6 to 1.25% (w/v) were tested. Three concentrations of poloxamer 188 were tested (1.25, 2.5 and 3% (w/v)). The Poloxamer 188/PL ratios ranged from 1 to 5. Pentamidine concentration was 0.5 mg/ml. Six formulations (A, B, C, D, E and F) were studied and represented in Table 1.

Unloaded nanoparticles were prepared according to the same formulation omitting pentamidine.

2.4. Physicochemical characterization of nanoparticles

2.4.1. Determination of drug loading

Total pentamidine concentration was determined after dissolution of nanoparticles in a mixture composed of acetonitrile (80%) and water (20%). Free pentamidine was determined after separation of loaded-nanoparticles from the aqueous medium by a combined ultrafiltration-centrifugation technique (Ultrafree MC®, Millipore, Bedford, TX). Ultrafiltrate was diluted in the same solvent mixture. The bound percentage (BP) was calculated as follows:

$$BP = [(\text{total pentamidine} - \text{free pentamidine})/\text{total pentamidine}] \times 100$$

Free and bound pentamidine were assessed by high-performance liquid chromatography. Briefly, each sample (100 μl) was injected onto a reverse-phase C_{18} column (Hypersil, 5 μm, 250 mm × 4.6 mm, I.D., Shandon, France). The mobile phase was composed of 700 ml of methanol and 300 ml of a 0.05 M of heptane-sulfonic acid and 0.014 M

of diethylamine aqueous solution. The pH of the aqueous solution was adjusted to 3.00 using phosphoric acid. The flow rate was 0.9 ml/min. Detection of pentamidine was performed by ultraviolet absorption at 280 nm. Peak area was used to determine pentamidine concentrations.

2.4.2. Photon correlation spectroscopy

Particle size distribution, average size and polydispersity index were measured by laser scattering using a monochromatic laser ray diffusion counter (Nanosizer N4, Coultronics, Margency, France).

2.4.3. Determination of pH

The pH of nanoparticle suspensions was determined at 20°C using a digital pH meter 646 (Prolabo, Paris, France).

2.4.4. Determination of nanoparticle suspension absorbance

The nanoparticle suspension was diluted in water (1/20) and the optical density was measured at 510 nm.

2.4.5. Scanning electron microscopy (SEM)

Aqueous dispersion of nanoparticles (unloaded nanoparticles and pentamidine loaded nanoparticles (formulation E) were finely spread over a slide and dried under vacuum. The sample was shadowed in a cathodic evaporator with a gold layer (20 nm thick). Then, the surface morphology of the nanoparticles was observed by SEM using a JSM 840 A scanning electron microscope (JEOL, Tokyo, Japan).

2.5. Stability study

The effects of storage time (0–9 months) on pH, particle size, drug loading and absorbance of nanoparticles were investigated. The suspensions were stored at 4°C and protected from light. The effect of storage on morphological form was investigated over 1 year with unloaded and pentamidine loaded nanoparticles (formulation E).

All assays were performed in quadruplicate.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation. An one-way analysis of variance or a U-test was performed to compare the influence of the various parameters. A p-value less than 0.05 was considered as representing a significant difference.

3. Results and discussion

3.1. Physicochemical characterics of pentamidine loaded nanoparticles

Pentamidine-loaded nanoparticles were easily obtained with all formulations tested. Nevertheless, the percentage of pentamidine binding was different and dependent on the PL concentration. The highest binding percentage (75.8% ± 1.8) was obtained with the highest concentration of PL (1.25%). Under these conditions, 379 μg was the maximal pentamidine amount loaded per ml of suspension. A linear regression between the bound pentamidine and the PL concentration ($r = 0.991$) was found (Fig. 1). The increase of PL or pentamidine concentration was impossible because the low solubility of PL in acetone and the low solubility of pentamidine in ethanol and in acetone. Therefore, 0.5 mg/ml was found to be the maximal solubility of pentamidine in ethanol. For

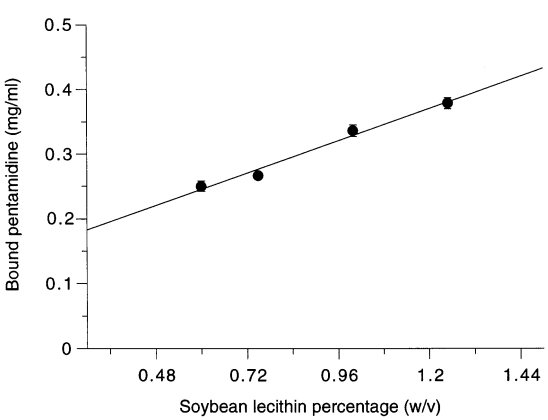

Fig. 1. Influence of the soybean lecithin percentage on amounts of pentamidine bound to PLA nanoparticles.

Poloxamer 188/PL ratios superior to 2.5, the percentage of binding was significantly lower than those obtained with the E and F formulations (ratios = 2 and 1, respectively).

The colloidal suspensions were monodispersed with a mean diameter of between 131 and 154 nm (Table 2). This diameter was similar to the one obtained with primaquine-loaded poly(lactide) nanoparticles (Rodrigues et al., 1995). The nature and amount of surfactants are important factors for the size distribution (Magenheim and Benita, 1991), but in our study, the Poloxamer 188/PL ratio did not influence the nanoparticle size. Unloaded nanoparticles (formulation E) presented a similar mean diameter (128 ± 15.2 nm). The fixation of pentamidine on poly(lactide) polymers did not modify the mean particle size. These results comfirm those obtained with primaquine on PIHCA (Gaspar et al., 1991). The electron micrographs showed spherical discrete and homogenous particles in the nanometer size range (Fig. 2). Similar micrographs were obtained whatever the formulation tested.

To improve the fixation yield, the preparation was realized under alkaline conditions. As demonstrated by Rodrigues, an alkalin pH allowed to increase the percentage of primaquine fixation to poly(lactide) nanospheres (Rodrigues et al., 1995). The pH of different formulations was comprised between 7.5 and 8 (Table 2). Unloaded nanoparticles (formulation E) had a pH significantly higher than pentamidine-loaded nanoparticles (8.3 ± 0.04 vs. 8.0 ± 0.05). As ammoniac is a stronger base than pentamidine, pH of unloaded nanoparticles is higher than that of loaded nanoparticles.

The optical density at 510 nm was performed to appreciate the turbidimetry. For Poloxamer 188/PL ratios superior to 2.5, a significant increase in optical density at 510 nm was observed. An exponential relation was found between the absorbance of colloidal suspensions and the PL concentration ($r = 0.997$) (Fig. 3). As in our study, particle diameters and polymer concentration were not significantly different fron one formulation to the other, the increase in optical density was probably due to the presence of non entrapped PL in the medium.

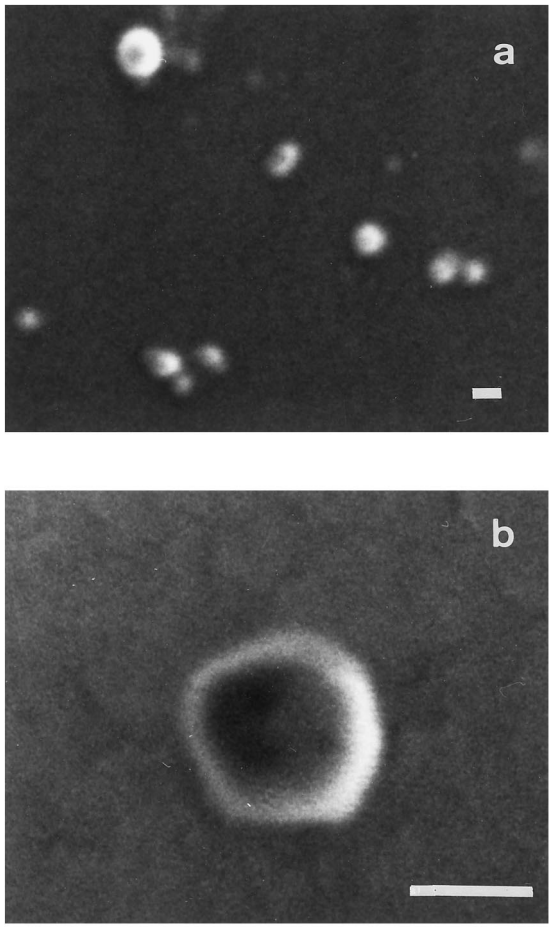

Fig. 2. SEM micrograph of PLA nanoparticles prepared according to the formulation F: (a) unloaded nanoparticles; (b) pentamidine-loaded nanoparticles. Scale bar = 0.1 μm.

3.2. Stability studies

All formulations were stable after a period of 9 months of storage at 4°C. No precipitation or aggregation was observed. Table 3 summarizes the physicochemical characteristics of the different formulations tested. The percentage of binding was significantly lower for formulations (B, C, E and F). A release of between 12 and 21% was observed. To describe the drug release from nanoparticles, an empirical equation was used (Ritger and Peppas, 1987) (Fig. 4):

$$M_t/M_0 = Kt^n \quad (1)$$

Table 2
Physicochemical characteristics of different formulations

Formulation	A		B		C		D		E		F
Percentage of binding	48.1 ± 2.5	§	50.1 ± 1.6	§	53.4 ± 0.9	§	67.3 ± 1.7	*	75.8 ± 1.8		73.9 ± 2.1
Size (nm)	132 ± 12.4		139 ± 9.2		154 ± 12.9		142 ± 12.8		131 ± 15.6		133 ± 16.0
pH	7.5 ± 0.10	*,○	7.8 ± 0.07	*	7.5 ± 0.05	*,○,§	7.9 ± 0.10		8.0 ± 0.05	*	7.8 ± 0.06
Do (510 nm)	0.10 ± 0.01	○,§	0.12 ± 0.01	○,§	0.14 ± 0.02	*,○	0.19 ± 0.01	*	0.28 ± 0.03		0.21 ± 0.01

Results are expressed as mean $\pm$ S.E.
* $p<0.05$; ○ $p<0.01$; § $p<0.001$.
Percentage of binding: A vs. D, E and F; B vs. D, E and F; and C vs. D, E and F: $p<0.001$. D vs. E: $p<0.05$.
Do (510 nm): A vs. D and F; and B vs. F: $p<0.001$. A vs. E; B vs. D and E; C vs. E:. C vs. F; and D vs. E: $p<0.05$.
pH: A vs. B, D and F; B vs. C; and C vs. D: $p<0.05$. A vs. E; and C vs. F: $p<0.01$. C vs. E: $p<0.001$.

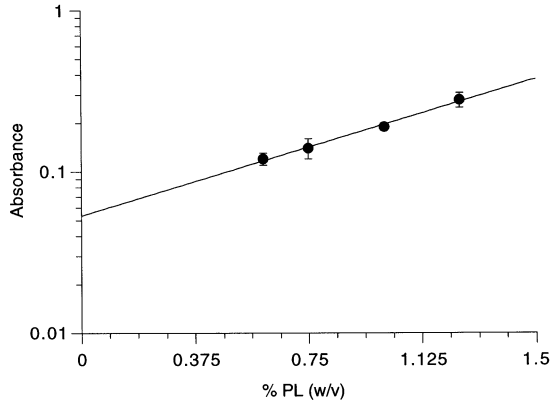

Fig. 3. Influence of the soybean lecithin percentage on optical density of nanoparticle suspensions.

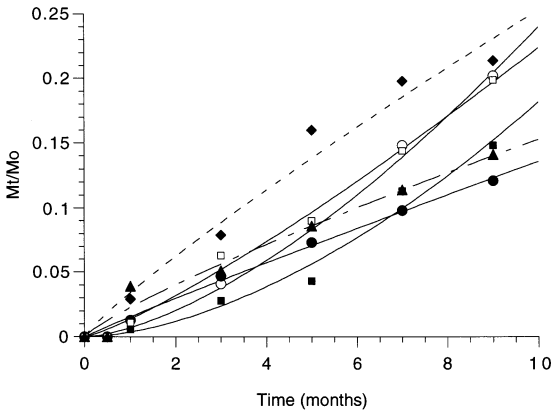

Fig. 4. Plots of ratio (amount of drug release at time t/initial drug content) vs. time. Formulations were stored in water at 4°C, over 9 months: ■, formulation A; ○, formulation B; ◆, formulation C; ●, formulation D; □, formulation E; ▲, formulation F.

where M_t is the amount of drug released at time t; M_0 the initial drug content; n the diffusional exponent; K the constant including structural and geometric characteristics.

Exponential dependence of the amount of drug release (M_t/M_0) on time was evidenced ($r > 0.98$). For all formulations, the diffusional exponent was superior to 0.5 (0.8–1.7) indicating an anomalous diffusion and suggesting diffusion through the matrix. This model is usually used to describe the release kinetics at 37°C in phosphate buffer saline. But, at 4°C, the molecular interactions were very slow, explaining an anomalous diffusion. An anomalous release was also found with swellable devices as nanoparticles (Ritger and Peppas, 1987). The in vitro release profile from nanoparticles can also be describe using another empirical mathematical expression (Illum et al., 1986):

$$M_0 - M_t/M_0 = A\ \mathrm{e}^{-\alpha t} + B^{-\beta t} \quad (2)$$

where A and B are constants; α and β are rate constants for the initial and late time releases.

In our study, a mono-exponential equation was found ($r > 0.97$), probably due to the slow release at 4°C (Fig. 5). It was also reported that polymer degradation followed a first order kinetics at 37°C (Lemoine et al., 1996). The pentamidine release

Table 3
Stability studies: physicochemical characteristics of different formulations after a storage of 9 months at 4°C

Formulation	A		B		C		D		E	F
Percentage of binding	41.0 ± 2.0	○,§	40.0 ± 1.5	○,§	42 ± 1.0	○,§	59.2 ± 3		63.2 ± 2.2	62.6 ± 1.8
Size (nm)	121 ± 9.3	*	132 ± 14.6		154 ± 13.0		164 ± 14.5		133 ± 13.6	141 ± 12.0
pH	5.6 ± 0.10	*,○	5.7 ± 0.12	*,§	5.5 ± 0.10	○	5.6 ± 0.10	*,○	6.7 ± 0.20	6.0 ± 0.15
Do (510 nm)	0.17 ± 0.02	§	0.18 ± 0.02	§	0.20 ± 0.02	○,§	0.42 ± 0.03	○	0.46 ± 0.04	0.20 ± 0.03

Results are expressed as mean ± SE.
* $p < 0.05$; ○ $p < 0.01$; § $p < 0.001$.
Percentage of binding: A vs. D; B vs. D; and C vs. D: $p < 0.01$. A vs. E and F; B vs. E and F; and C vs. E and F: $p < 0.001$.
pH: A vs. F; F vs. F; and D vs. F: $p < 0.05$. A vs. E; B vs. E; C vs. E and F; and D vs. E: $p < 0.01$.
Do (510 nm): A vs. D and E; B vs. D and E; and C vs. D: $p < 0.001$. C vs. E; and D vs. E and F: $p < 0.01$.

should obey the same mathematical equation than the polymer degradation. Moreover, the degradation rate constants derived from the logarithmic plots of molecular weight/initial molecular weight of PLA50 (Lemoine et al., 1996) were similar to these obtained from the mono-exponential equation (0.026 $month^{-1}$ vs. 0.022 $month^{-1}$). A stability assay was performed on the primaquine-loaded poly(lactide) nanoparticles over a period of 3 months. No significant release was observed over this period (Rodrigues et al., 1995). Another study, conducted at 20°C, with poly(lactide) nanocapsules containing diclofenac demonstrated a good stability after 8 months of storage (Guterres et al., 1994). Actually, PLA nanoparticles show a slow degradation of the polymer when kept at 4°C and room temperature. At 37°C, the polymer is degraded more quickly (Lemoine et al., 1996).

The pH of nanoparticle suspensions significantly decreased after 9 months of storage. The mean decrease in pH reached about 2 units for all formulations except for the E formulation. A linear regression was found between pH and time ($r > 0.99$) (Fig. 6). As the polymer degradation follows a first order kinetics and the pH is a logarithmic function of the proton concentration, it was consistent to find a linear equation. The other studies with PLA polymer also demonstrated a decrease of the pH (Grizzi et al., 1995; Guterres et al., 1994; Lemoine et al., 1996). Nevertheless, the pH decrease was lower than the one obtained in our experiments (1 unit of pH in 8–12 months). This difference could be explained by the initial pH of the nanoparticles suspensions. Usually, in these studies, the initial pH was lower (pH between 4 and 5). At alcalin pH, the degradation of PLA polymer was accelerated (Makino et al., 1985). The accelerated decrease of pH could also be explained by the presence of the pentamidine. Lin et al. have demonstrated that the organic amines enhanced the polyester polymer degradation (Lin et al., 1994).

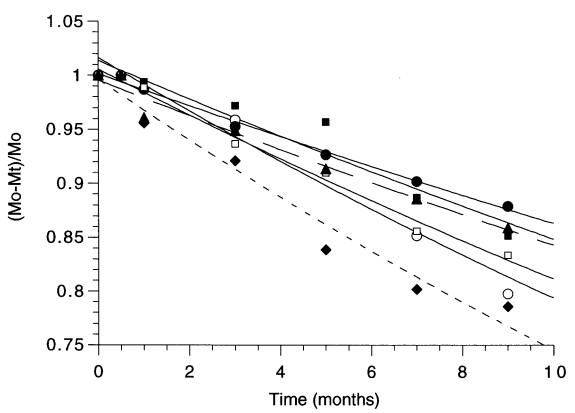

Fig. 5. Plots of ratio [(initial drug content × amount release)/initial drug content] vs. time. Formulations were stored in water at 4°C, over 9 months: ■, formulation A; ○, formulation B; ◆, formulation C; ●, formulation D; □, formulation E; ▲, formulation F.

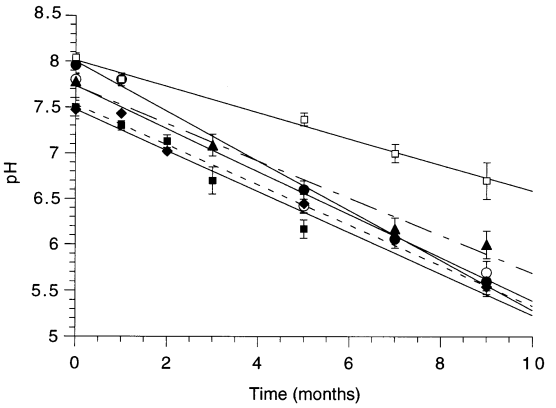

Fig. 6. Influence of time on pH of nanoparticle suspensions stored in water at 4°C, over 9 months: ■, formulation A; ○, formulation B; ◆, formulation C; ●, formulation D; □, formulation E; ▲, formulation F.

Nanoparticle size was not significantly modified after 9 months of storage and suspensions stayed always monodispersed. Coffin and McGinity have found that the degradation of polyester polymer was very slight after 1 year at 4°C (Coffin and McGinity, 1992). In fact, degradation of nanoparticles is heterogenous and proceeds more rapidly in the centre than on surface (Park, 1995). Nanoparticles kept their initial form without surface modification until 2 years at 4°C (Fig. 7). These results confirm those obtained by scanning electron microscope with PLA microspheres (Grizzi et al., 1995). Another study demonstrated that microspheres stored at 37°C, in phosphate buffer saline, kept their initial morphology for 6 weeks (Spenlehauer et al., 1989).

The optic density significantly increased for all formulations except for the F formulation. The multiplicative factor was about 1.7. This increase was not corroborated to an increase of the size. The presence of surfactants (PL and poloxamer 188) in the media could induce an increase of the opalescence.

The nanoprecipitation method allowed to obtain easily pentamidine-loaded (D,L-lactide) nanoparticles with a good fixation yield particularly for the formulation associating high concentrations of PL and the poloxamer in a ratio 1/2. These nanoparticles were stable and can be administered to the *Leishmania* infected mice to evaluate their interest in the treatment of visceral leishmaniasis.

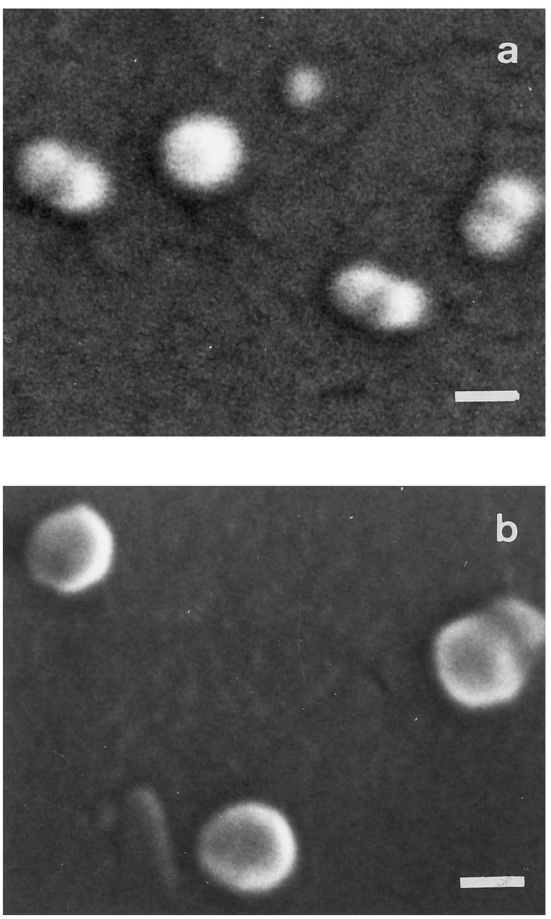

Fig. 7. SEM micrograph of PLA nanoparticles prepared according to the formulation F after 1 year at 4°C: (a) unloaded nanoparticles; (b) pentamidine loaded nanoparticles. Scale bar = 0.1 μm.

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References

Alving, C.R., Steck, E.A., Hanson, W.L., Loizeaux, P.S., Chapman, W.L., Waits, V.B., 1978. Improved therapy of experimental leishmaniasis by use of a liposome encapsulated antimonial drugs. Life Sci. 22, 1021–1026.

Alving, C.R., 1986. Liposomes as drug carriers in leismaniasis and malaria. Parasitol. Today 2, 101–107.

Berman, J.D., Gallalee, J.V., Williams, J.S., Hockmeyer, W.D., 1986. Activity of pentamidine-containing human red cell ghosts against visceral leishmaniasis in the hamster. Am. J. Trop. Med. Hyg. 35, 297–302.

Bazile, D.V., Ropert, C., Huve, P., Verrecchia, T., Marland, M., Frydman, A., Veillard, M., Spenlehauer, G., 1992. Bodydistribution of fully biodegradable 14C-poly(lactic acid) nanoparticles coated with albumin after parenteral administration to rats. Biomaterials 13, 1093–1102.

Coffin, M.D., McGinity, W., 1992. Biodegradable pseudolatexes: the chemical stability of poly(D,L-lactide) and poly(ε-caprolactone) nanoparticles in aqueous media. Pharm. Res. 9, 200–205.

Croft, S.L., Neal, R.A., Rao, L.S., Liposomes and other drug delivery systems in the treatment of leishmaniasis. In: Hart D.T. (Ed.), Leishmaniasis: The Current Status and New Strategies for Control. Plenum Press, New York, 1989, pp. 111–118.

Davidson, R.N., Croft, S.L., Scott, A., Maini, M., Moody, A.H., Bryceson, A.D.M., 1991. Liposomal amphotericin B in drug-resistant visceral leishmaniasis. Lancet 337, 1061–1062.

Davidson, R.N., Croft, S.L., 1993. Recent advances in the treatment of visceral leishmaniasis. Trans. R. Soc. Trop. Med. 87, 130–131.

Davidson, R.N., Di Martino, L., Gradoni, L., Giacchino, R., Gaeta, G.B., Pempinello, R., Scotti, S., Cascio, A., Castagnola, E., Maisto, A., Gramiccia, M., Caprio, D.Di, Wilkinson, R.J., Bryceson, A.D.M., 1996. Short-course treatment of visceral leishmaniasis with liposomal amphotericin B (Ambisome). Clin. Infect. Dis. 22, 938–943.

Debs, R.J., Brunette, E.N., Lin, J.M., Lin, E.J., Montgomery, A.B., Friend, D.S., Pahahadjopoulos, D.P., 1987. Selective enhancement of pentamidine uptake in the lung by aerosolization and delivery in liposomes. Am. Rev. Respir. Dis. 135, 731–737.

Deniau, M., Durand, R., Bories, C., Paul, M., Astier, A., Couvreur, P., Houin, R., 1993. Etude in vitro de médicaments leishmanicides vectorisés. Ann. Parasitol. Hum. Comp. 68, 34–37.

Durand, R., Paul, M., Rivollet, D., Houin, R., Astier., A., Deniau, M., 1997. Activity of pentamidine-loaded methacrylate nanoparticles against *Leishmania infantum* in a mouse model. Int. J. Parasitol. 87 (11), 1363–1367.

Fessi, H., Devissaguet, J.P., Puisieux, F., Thies, C., 1992. Process for preparation of dispersible colloidal systems of a substance in the form of nanoparticles, US Patent no. 5 118 528.

Fusaï, T., Deniau, M., Durand, R., Bories, C., Paul, M., Rivollet, D., Astier, A., Houin, R., 1994. Action of pentamidine bound nanoparticles against *Leishmania* on an in vivo model. Parasite 1, 319–324.

Gaspar, R., Préat, V., Roland, M., 1991. Nanoparticles of polyisohexylcyanoacrylate (PIHCA) as carriers of primaquine: formulation, physicochemical characterization and acute toxicity. Int. J. Pharm. 68, 111–119.

Gaspar, R., Opperdoes, F.R., Préat, V., Roland, M., 1992. Drug targeting with polyalkylcyanoacrylate nanoparticles: in vitro activity of primaquine-loaded nanoparticles against intracellular *Leishmania donovani*. Ann. Trop. Med. Parasitol. 86, 41–49.

Grizzi, I., Garreau, H., Li, S., Vert, M., 1995. Hydrolytic degradation of devices based on poly(D,L-lactic acid) size-dependence. Biomaterials 16, 305–311.

Guterres, S.S., Fessi, H., Barrat, G., Devissaguet, J.P., Puisieux, F., 1994. Poly(D,L-lactide) nanocapsules containing diclofenac: formulation and stability study. Int. J. Pharm. 113, 57–63.

Illum, L., Khan, M.A., Mak, E., Davis, S.S., 1986. Evaluation of carrier capacity and release characteristics for poly(butyl 2-cyanoacrylate) nanoparticles. Int. J. Pharm. 30, 17–28.

Kulkarni, R.K., Pani, K.C., Neuman, C., Leonard, F., 1966. Biodegradable poly(lactic acid) polymers. J. Biomed. Mater. Res. 5, 839–843.

Kulkarni, R.K., Moore, E.G., Hegyeli, A.F., Leornarde, F., 1971. Biodegradable poly(lactic acid) polymers. J. Biomed. Mater. Res. 5, 169–181.

Lemoine, D., Francois, C., Kedzierewicz, F., Preat, V., Hoffman, M., Maincent, P., 1996. Stability study of nanoparticles poly(ε-caprolactone), poly(D,L-lactide) and poly (D,L-lactide-co-glycolide).. Biomaterials 17, 2191–2197.

Lin, W.J., Flanagan, D.R., Linhardt, R.J., 1994. Accelerated degradation of poly(ε-caprolactone) by organic amines. Pharm. Res. 11, 1030–1034.

Magenheim, B., Benita, S., 1991. Nanoparticle characterization: a comprehensive physicochemical approach. STP Pharm. Sci. 1, 221–241.

Makino, K., Arakawa, M., Kondo, T., 1985. Preparation and in vitro degradation properties of polylactide microcapsules. Chem. Pharm. Bull. 33, 1195–1201.

Modabber, F., Leishmaniasis. In: Maurice, J., Pierce, A.M. (Eds.), Tropical Disease Research progress 91–92, 11th Program Report. World Health Organization, Geneva, 1992, pp. 77–81.

New, R.R., Chance, M.L., 1980. Treatement of experimental cutaneous leishmaniais by liposome-entrapped pentostam. Acta Trop. 37, 253–256.

Park, T.G., 1995. Degradation of poply(lactic-co-glycolic acid) microspheres: effect of the copolymer composition. Biomaterials 16, 1123–1130.

Paul, M., Durand, R, Boulard, Y., Fusaï, T., Fernandez, C., Rivollet, D., Deniau, M., Astier, A., 1997. Physicochemical characteristcs of pentamidine-loaded polymethacrylate nanoparticles: implication in the intracellular drug release in *Leishmania major* infected mice. J. Drug. Target.

Puisieux, F., Barrat, G., Couarraze, G., Couvreur, P., Devissaguet, J.P., Dubernet, C., Fattal, Y., Fessi, H., Vauthier, C., 1994. Polymeric micro and nanoparticles as drug carriers. In: Severian Dimitriu (Ed.), Polymeric Biomaterials. Marcel Dekker, New York, 1994, pp. 749–794.

Ritger, P.L., Peppas, N.A., 1987. A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. J. Control. Release 5, 37–42.

Rodrigues, J.M. Jr., Croft, S.L., Fessi, H., Bories, C., Devissaguet, J.Ph., 1994. The activity and ultrastructural localization of primaquine-loaded poly(D,L-lactide) nanoparticles in *Leishmania donovani* infected mice. Trop. Med. Parasitol. 45, 223–228.

Rodrigues, J.M. Jr., Fessi, H., Bories, C., Puisieux, F., Devissaguet, J.Ph., 1995. Primaquine-loaded poly(lactide) nanoparticles: physicochemical study and acute tolerance in mice. Int. J. Pharm. 126, 253–260.

Rolland, A., 1987. Mise au point et applications de nanospheres á base de copolymères methacryliques. Intérêt pour la vectorisation d'agents cytostatiques (anthracyclines). Ph.D. Thesis, Université de Rennes, France.

Spenlehauer, G., Vert, M., Benoit, J.P., Boddaert, A., 1989. In vitro and in vivo degradation of poly(D,L-lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials 10, 557–563.

Torre-Cisneros, J., Villanueva, J.L., Kindelan, J.M., Jurado, R., Sanchez-Guijo, P., 1993. Successful treatment of antimony-resistant visceral leishmaniasis with liposomal amphotericin B in patients infected with human immunodeficiency virus. Clin. Infect. Dis. 17, 625–627.

Venier-Julienne, M.C., Vouldoukis, Monjour L., Benoit, J.P., 1995. In vitro of the antileishmanial activity of biodegradable nanoparticles. J. Drug. Target 3, 23–29.

Youssef, M., Fattal, E., Couvreur, P., Adremont, A., 1988. Treatment of experimental salmonellosis in mice with ampicillin-bound nanoparticles. Antimicrob. Agents Chemother. 33, 1204–1207.