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Molecular Recognition by *Kluyveromyces* of Amphotericin B-Loaded, Galactose-Tagged, Poly (Lactic Acid) Microspheres

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Abstract—In an effort to develop a new way of drug delivery, especially for polyenic antifungal molecules, we have incorporated amphotericin B (AmB) into biodegradable galactosylated poly (L-lactic acid) (L-PLA) and poly (L-lactic-co-glycolic acid) (PLGA) microspheres. These drug carriers were prepared by solvent evaporation using an oil/water (o/w) emulsion. The ratio of galactosyl spacers with different chain lengths was 1.74–2.78%. The maximal quantity of AmB encapsulated reported to 100 mg of the galactosylated microspheres was 7.14 mg for L-PLA (encapsulation rate 45% of mole) and 6.42 mg for PLGA derivatives (encapsulation rate 81% of mole). In our yeast model, drug release depended on three factors: (i) presence of galactosylated antennae, (ii) length of galactosyl antenna and (iii) nature of the polymer. More of the AmB trapped in PLGA microspheres was released than from PLA microspheres. These novel functionalised microspheres could be required for the delivering of therapeutic agents according to their recognition to specific cells.

Introduction

Amphotericin B (AmB) is still considered the gold standard of antifungal drugs because of its great therapeutic efficacy. This member of the macrolide polyenic family of antibiotics has been formulated as a self-micellar system.[1] Nevertheless, the major problem with this drug is its great acute toxicity, its instability and its insolubility in water. Drug delivery systems such as (nano and micro) particles have been developed to avoid these problems. These systems protect drugs against breakdown[2] and reduce adverse effects.[3]

The choice of vector depends on the lipophilic character of drugs, which assumes that these molecules could be delivered by lipidic vectors, micro or nano particles or in the hydrophobic cavity of a supramolecular system. Rajagopalan et al.[4] used γ-cyclodextrin as a carrier for AmB. AmB has also been encapsulated in nanoparticles of poly (L-lactide-co-glycolic acid) (PLGA),[5] however the inclusion rate determined no exceed 2%. Tabosa Do Egito et al.[6] have recently developed a new system for AmB based on a lipid emulsion with high concentration of AmB (1 mg/mL) (Fig. 1).

Poly (lactic acid) (PLA) derivatives are widely used in a variety of medical applications, such as for bonding fractures[7] in drug delivery systems,[8] and as resorbable sutures.[9] Poly (L-lactic acid) (L-PLA) and poly (L-lactic-co-glycolic acid) (PLGA) are now widely used in pharmaceutical formulations because of their biodegradability, their low toxicity and good mechanical properties.[10] Microparticles are widely employed in medical science, such as in drug targeting, and they are one of the most desirable vectors.[11] Several groups have looked at the encapsulation of bioactive molecules in microspheres based on PLA.[12–14] In spite of the variety of applications of nanoparticles, microspheres are useful

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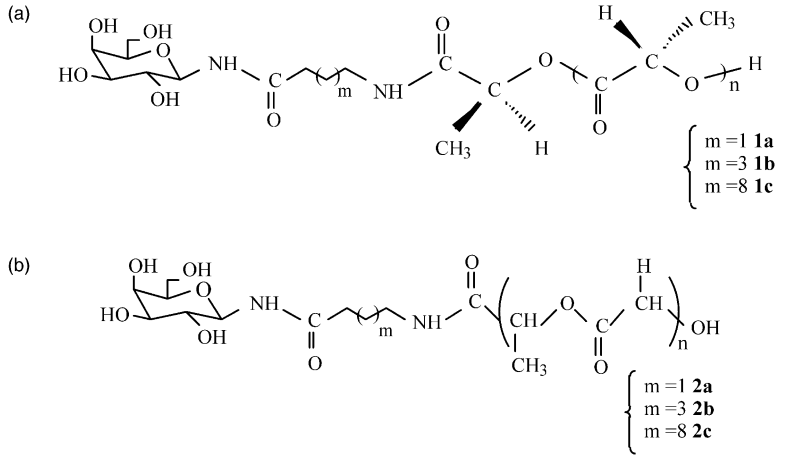

Figure 1. Structure of L-PLA (A) and PLGA (B) galactosyl derivatives.

to provide a maximum rate of drug encapsulation, allowing their controlled release. Many studies have been to formulate and characterise a controlled release biodegradable delivery system of drugs for the treatment or prevention of diseases. So, poly (DL-lactic acid) microspheres containing glucocorticoids such as dexamethasone can facilitate mucosal repair in experimental colitis and could be an ideal agent for treatment of human inflammatory bowel disease.[15] Similar biodegradable polymers as PLGA containing guanosine have been prepared as model drug for intraocular administration.[16] Microspheres of progesterone have been formulated using copolymers of PLGA and L-PLA, and should provide a means of delivering progesterone for months for the treatment or prevention of osteoporosis in postmenopausal women.[17] It has been shown that the reversibility of binding between RCA_{120} lectin and lactose-immobilized gold nanoparticles was confirmed by addition of free galactose, and observe the particle surface plasmon band indicate the dissociation of gold nanoparticles cross-linked with RCA_{120} lectin.[18,19]

We have attempted to improve the administration of AmB, L-PLA and PLGA galactosyl microspheres by developing a solvent evaporation method. These polymers must be made by a galactosyl antenna possessing spacer chains of different lengths so as to target drugs to cells bearing galactose-specific receptors at their surface. We have demonstrated that coupling carbohydrates directly to a vector lead to a good molecular recognition by the cell wall galactose lectins of *Kluyveromyces bulgaricus*.[20–22]

The present work concerns the study of the presence and accessibility of antennae for molecular recognition at the surface of microspheres. If the hypothesis that such antennae are, indeed, surface localised and remain presented after the preparation of the microspheres than the polymers described in this publication may serve for the formulation of colloïdal systems compatible with oral administration, and capable of vectoring pathogenic systems (e.g., *Candida* and *Aspergillus* species).

We now report the formulation of these galactosylated L-PLA and PLGA derivatives in the form of microspheres and demonstrate the capacity of these functionalised microspheres to encapsulate AmB and to be specificaly recognized by *K. bulgaricus* yeasts which bear galactose specific lectin and not by *Saccharomyces cerevisiae* yeasts which bear mannose specific lectin.

Table 1. Specification of L-PLA and PLGA derivatives

Product[a]	$\overline{M_w}$ (Daltons)	$\overline{M_n}$ (Daltons)	I
L-PLA	73,000	32,000	2.2
1a	76,200	48,600	1.5
1b	77,800	44,300	1.75
1c	76,600	42,500	1.80
PLGA	34,000	21,000	1.6
2a	28,000	17,000	1.6
2b	29,000	17,500	1.7
2c	27,000	16,500	1.6

[a]Product numbers refer to Figure 1.

Results

Molecular weights

The GPC measurements show a change in $\overline{M_w}$ and $\overline{M_n}$ after attaching the galactosylated antennae. Change may be explained by adding $\overline{M_n}$ chains. The decrease in $\overline{M_n}$ for PLGA may be due to hydrolysis of the microspheres during analysis (Table 1).

Size and encapsulation rate of AmB into microspheres

We determined the quantity required to prepare control (without antennae and drug) L-PLA microspheres to access the influence of stirring speed and surfactant concentration on the size of the microspheres. The microsphere diameter decreased when either the agitation speed or the surfactant concentration was increased (Table 2).

The microspheres must be small for oral administration or vectorisation. We used a stirring speed of 700 rpm and a surfactant concentration of 18% to prepare galactosyl antennae functionalised microspheres. These microspheres were prepared by oil/water (o/w) emulsion

solvent evaporation and had an uniform distribution and a diameter of 100–300 μm (Table 3).

The percentage of these galactosylated antennae was 1.74–2.78% by titration of these new polymer derivatives with 0.01 N methanolic sodium solution.[23] Titration of the L-PLA-Gal derivatives **1a**, **1b** and **1c** shows, respectively, the presence of 3.5×10^{-6}, 3.0×10^{-6} and 4.0×10^{-6} carboxylic acid functions per 100 mg. Titration of the 100 mg of L-PLA shows the presence pf 10^{-5} carboxylic functions. The quantity of antennae functions coupled are thus 6.5×10^{-6} mol (1.74 mg) for **1a**, 7.0×10^{-6} mol (2.04 mg) for **1b** and 6.0×10^{-6} mol (2.78 mg) for **1c**, the figures in parentheses being weights per 100 mg. We determined the highest ratio of AmB entrapment in these functionalised microspheres by adding increasing quantities of AmB during preparation. The maximal percentage of encapsulated AmB was obtained when 80 and 40 mg of **1c** and **2c** were used, respectively. Application of the Beer–Lambert equation ($\varepsilon = 101{,}180$ cm^{-1} mol^{-1} L) gave the highest AmB encapsulation ratio of mole into L-PLA derivative microspheres as 45% for **1c**, 37% for **1b** and 34% for **1a**. The inclusion percentage defined as the quantity of AmB (X mol L^{-1}) encapsulated reported to 100 mg microspheres (Y mol L^{-1}). The encapsulation ratio was significantly improved with the PLGA derivative microspheres, giving encapsulation efficiencies of 81% for **2c**, 75% for **2b** and 68% for **2a** (Table 4). The maximal quantity of AmB encapsulated reported to 100 mg of the galactosylated microspheres was 7.14 mg for L-PLA and 6.42 mg for PLGA derivatives.

Table 2. Diameter of control L-PLA microspheres determined by light scattering

Condition		Diameter
% Tween	80	285
(speed: 700 rpm)	12	264
	18	194
Speed (rpm)[a]	400	280
(% Tween 80: 18)	500	243
	600	130
	700	129

[a]Results expressed in μm; standard deviation ± 28 μm.

Table 3. Size of loaded and unloaded (L-PLA and PLGA) galactosylated microspheres

Products	L-PLA	**1a**	**1b**	**1c**	PLGA	**2a**	**2b**	**2c**
Unloaded microspheres[a]	145	207	210	271	130	216	230	275
Microspheres + AmB[a]	168	213	238	147	149	205	216	187

[a]Results expressed in μm; standard deviation ± 28 μm.

Table 4. Percentage maximal encapsulation of AmB within L-PLA and PLGA galactosylated microspheres

Products	**1a**	**1b**	**1c**	**2a**	**2b**	**2c**
% Encapsulation AmB	34	37	45	68	75	81

Microscopy analysis

The external surfaces of L-PLA-Gal and PLGA-Gal microspheres were examined by atomic force microscopy (contact mode).

This instrument was chosen because the microspheres can be seen without using conventional methods commonly used in electron microscopy (EM). The microspheres were fixed on adhesive tape and directly

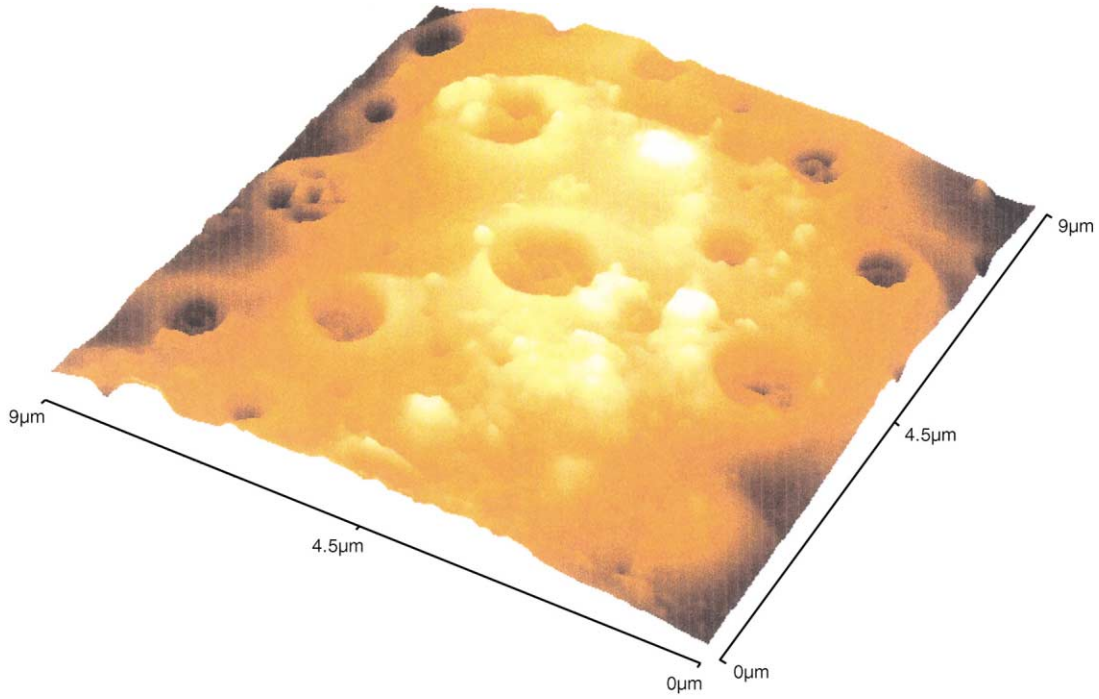

Figure 2. Microporous structure of microspheres observed by atomic force microscopy.

observed. Both types of microspheres gave reproducible images with no difference in the surface structural appearance with or without spacers (Fig. 2). Both types of microspheres were spherical with several cavities.

We attempted to demonstrate recognition of the microspheres by yeast cells by performing the assays in media that gave optimal yeast flocculation. The microsphere–yeast interaction was monitored under a light microscope. L-PLA-Gal microspheres were spheres of various sizes and were stable after 4 days under stirring in control liquid culture medium (Fig. 3a). These microspheres bound rapidly and avidly to *K. bulgaricus* flocculent cells (Fig. 3b). When *K. bulgaricus* yeasts are linked to the galactose tagged microspheres, these last are bursted after 24 h of incubation as shown in Fig. 3c. However, there appeared to be no binding to *S. cerevisiae* (Fig. 3d). These observations agree with the presence of galactose-specific lectins on *K. bulgaricus* surface. The assays also reveal that *S. cerevisiae* cells, which have no galactose-specific lectin at their surface, did not bind these microspheres.

Yeast–microsphere interaction

AmB must be released from the microspheres before it can be active, and this release depends on the chemical nature of the microspheres and on their activation, which may be triggered by the yeast.

Influence of the galactosylated antennae

The galactosylated antennae anchored to the microsphere surface were recognised by *K. bulgaricus* yeast cells. Culture medium (20 mL) containing 40 mg AmB encapsulated in microspheres (i) L-PLA + AmB microspheres, (ii) L-PLA-C11-Gal (**1c**) + AmB and (iii) the same quantity of AmB (40 mg) as that incorporated into the microspheres was inoculated with *K. bulgaricus* (2×10^7 cells/mL).

The presence of the galactosylated antennae on the microsphere delayed the growth of the *K. bulgaricus* yeasts for 32 h, while microspheres without galactosylated antennae delayed growth for 8 h (Fig. 4a). Unencapsulated AmB totally inhibited the growth of the yeasts. These results show the controlled release of AmB by the presence of galactosylated antennae. The lag time was 4 times longer than that for ungalactosylated microspheres. However, L-PLA-AmB-Gal and L-PLA-AmB microspheres (same quantity than that used for *K. bulgaricus*) did not modify the growth of *S. cerevisiae* (Fig. 4b).

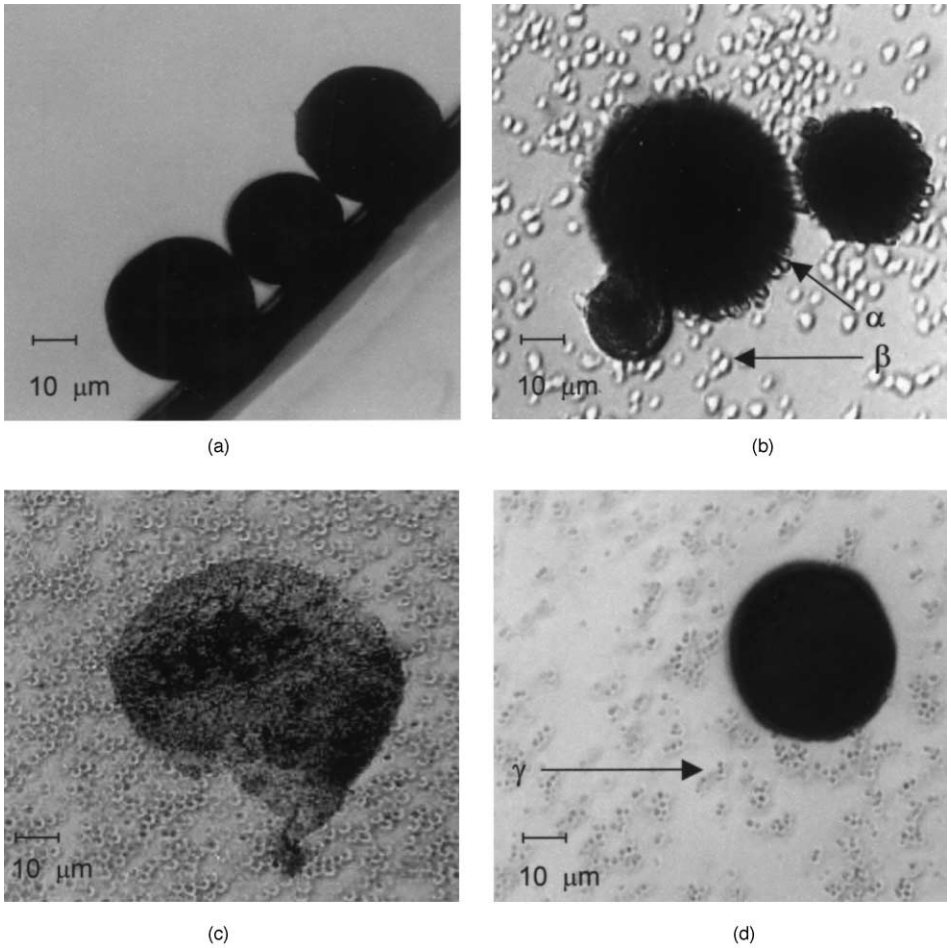

Figure 3. L-PLA-Gal microspheres under light microscope: (a): L-PLA-Gal microspheres; (b): L-PLA-Gal microspheres + *K. bulgaricus*: (α) yeast fixed on the microsphere; (β): yeast non fixed on the microsphere; (c) microsperes bursted after linkage to *K. bulgaricus;* (d) L-PLA-Gal microspheres + *S. cerevisiae:* (γ) yeast non fixed on the microsphere.

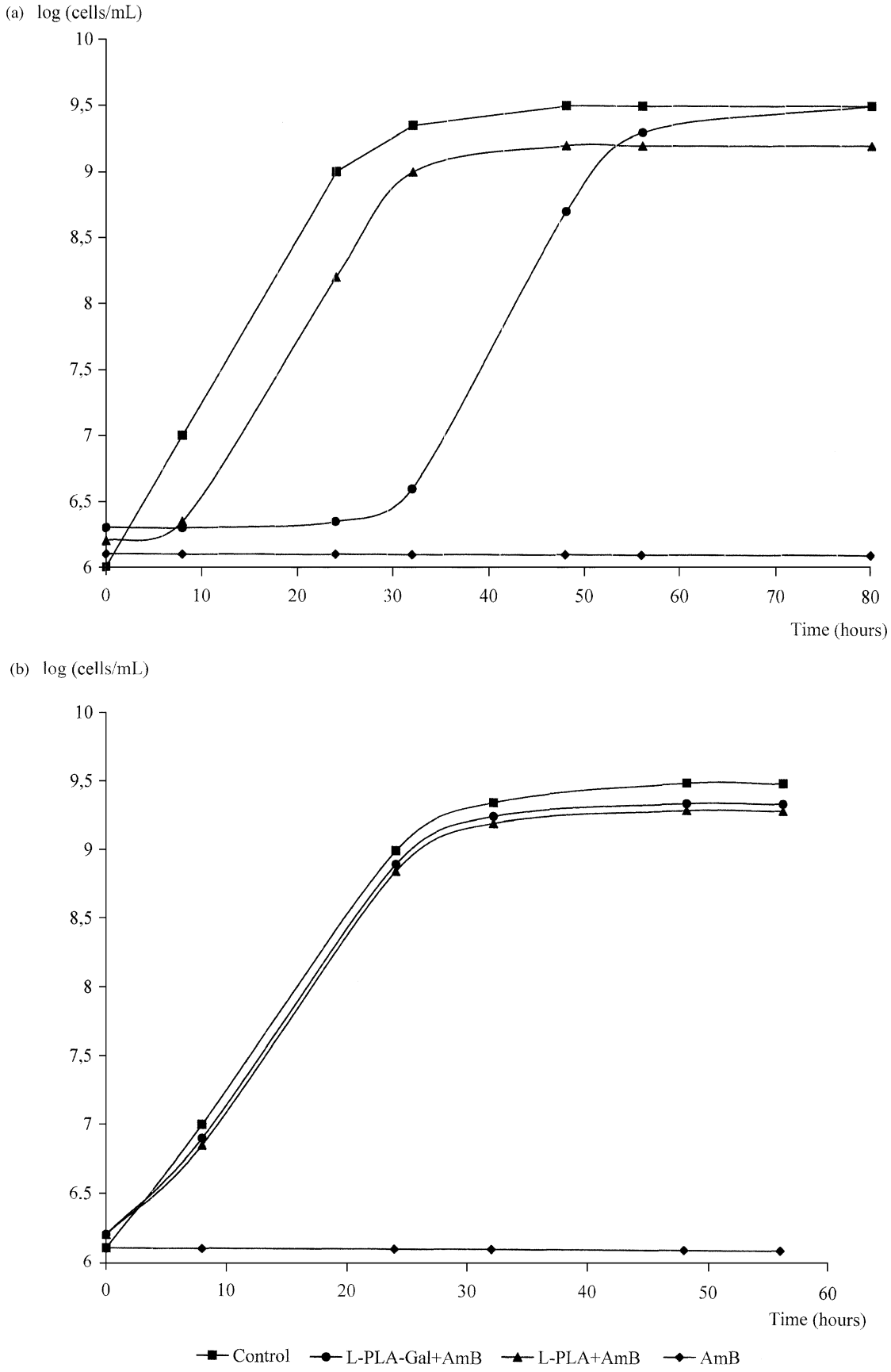

Figure 4. Influence of AmB encapsulated galactosyl antennae microspheres on the growth of the yeasts: (a): *K. bulgaricus*; (b) *S. cerevisiae*.

Influence of the length of the galactosylated antennae

The two microspheres studied were L-PLA-C6-Gal + AmB and L-PLA-C11-Gal + AmB, which differ by the length of the galactosylated antennae. L-PLA-C11-Gal + AmB microspheres inhibited the growth of *K. bulgaricus* better [30 h (±2)] than the L-PLA-C6-Gal + AmB [20 h (±2)] (Fig. 5). This difference may be due to a difference in the affinity of the galactose residue for the yeast lectins. A sugar far from the microsphere surface is more accessible to the yeast lectins. The hydrophobic property of the C11 chain may also enhance the affinity for the lectins on the yeast surface.

Influence of the nature of the polymer

The antifungal effect of AmB occurs only when it is released from the microspheres, and the growth rate of *K. bulgaricus* in culture medium was inhibited as a function of the AmB release. Free AmB at the same concentration as that encapsulated in microspheres was used as control. Assays with AmB trapped in PLGA-C11-Gal microspheres and with AmB trapped in L-PLA-C11-Gal microspheres demonstrated that the PLGA-C11-Gal significantly inhibited the growth of the yeast. The delay was 30 h (±2) with L-PLA-C11-Gal microspheres, and 48 h (±4) with PLGA-C11-Gal microspheres (**2c**) (Fig. 6).

This result was obtained with 0.005 g/L AmB encapsulated in PLGA microspheres, while 0.28 g/L AmB was encapsulated in L-PLA microspheres. These results indicate that PLGA microspheres were broken down faster and more completely than L-PLA microspheres. As the stabilities of the two types of microspheres were identical in various liquid media and buffer for 4 days under gentle stirring, the breakdown was due to the action of the yeasts by deconstruction of the PLGA and PLA and the role of yeast surface hydrolases (i.e., invertases) cannot be excluded.

Discussion

AmB is the most commonly used antifungal agent against systemic fungal infections. Its therapeutic delivery is limited by its toxicity against host eucaryotic cells which is also not well established. In an effort to direct its action against specific yeasts and fungi, this macrolide has been encapsulated. So, incorporation of AmB inside lipid structures (liposomes or lipid complexes as AmBisome™, Abelcet™) has provided encouraging in vitro and in vivo results. However, these formulations improve the application of the antibiotic by reduction of its toxicity.[24] So, to encapsulate hydrophobic molecules such as the polyenic antifungal agent AmB, the solvent evaporation method with an o/w system appeared as the

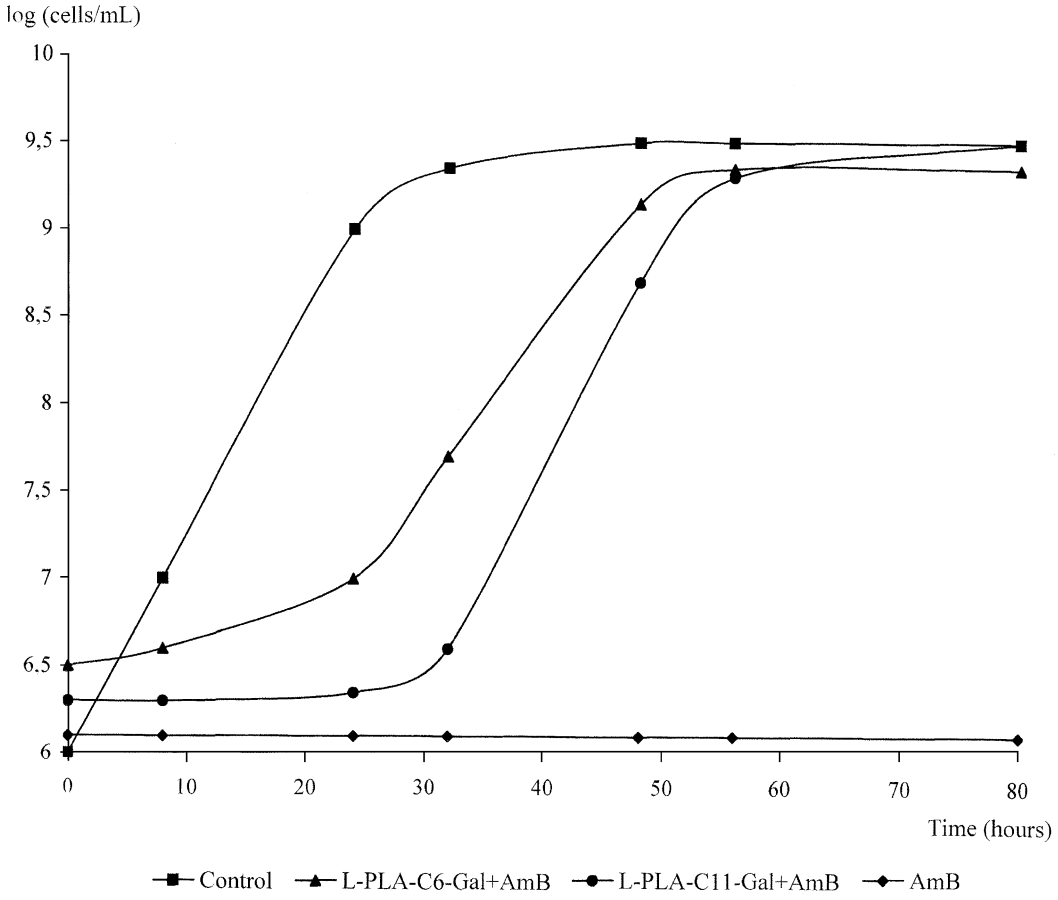

Figure 5. Influence of the length of the spacer of AmB encapsulated galactosylated antennae microspheres on the growth of *K. bulgaricus*.

most appropriate. Methylene chloride which has a low water solubility and low heat of evaporation, was the best solvent for preparing PLA microspheres by solvent evaporation. The resulting L-PLA and PLGA derivative microspheres had a homogenous distribution of particles. The difference in the microsphere size could be due to increased hydrophobic characteristics linked to the antennae incorporated into the polymeric chains (Table 2). The size of the microspheres depended on the stirring speed and the surfactant concentration. Examination of empty microspheres (145 ± 28 μm, 130 ± 28 μm), lacking the galactosyl antennae, showed that the spacers do not modify the properties of L-PLA and PLGA for the formation of such structures (271 ± 74 μm, 275 ± 74 μm). Maruyama et al.[25] have prepared particles bearing carbohydrate chains but the carbohydrate was not linked to the polymers, it was incorporated into the particles.

Atomic force microscope observations of the microspheres showed a microporous structure over all the surface. The cavities were produced during preparation, and their depth depended on the solvent evaporation conditions. The structure of the cavities remains unknown; however, these cavities do not allow the release of AmB in control culture media. Also, no relation can be established between microspheres–yeasts interactions and the surface microsporus structure. Futhermore, the maximum diameter of the cavities, that is 0.5–1 μm as estimated by AFM does not allow the incorporation of yeast cells inside the cavities of the microspheres.

The $\lambda = 409$ nm was used to determine the encapsulation rate of AmB. The maximal encapsulation was 45% for L-PLA-C11-Gal microspheres and 80% for PLGA-C11-Gal microspheres. This improved encapsulation rate could be due to a good affinity of AmB for PLGA.

We can assume that *K. bulgaricus* cells, which bear galactose-specific lectins, bound to the microspheres bearing galactosylated antennae, while *S. cerevisiae* cells, which have mannose-specific lectins, did not recognise the galactosylated antennae and did not bind to these microspheres.

The amphotericin B included in non-functionalised microspheres therefore had no or a low inhibitory effect on the growth of *Kluyveromyces* or *Saccharomyces*. However, when the microspheres bore galactose, they linked to *Kluyveromyces* yeasts bearing specific galactose-lectins. The microspheres bound to the yeasts were bursted near the linkage site (deconstruction of the PLGA or PLA or enzymatic hydrolysis probably), liberating the antifungal agent which then inhibited the growth of the yeasts. The same microspheres bearing galactose had no effect on the growth of *S. cerevisiae* yeasts which bear mannose-specific lectins.

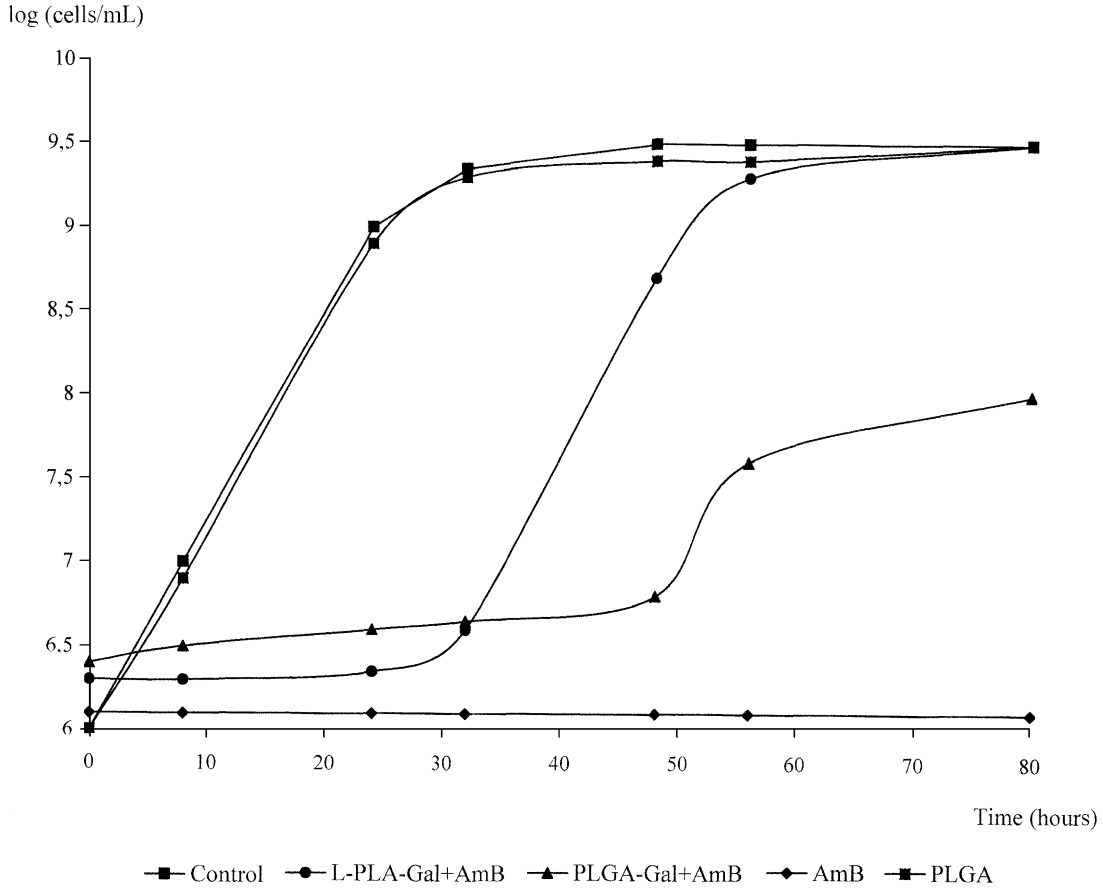

Figure 6. Influence of the nature of the polymer (L-PLA or PLGA) of AmB encapsulated galactosylated antennae microspheres on the growth of *K. bulgaricus*.

The presence of galactosylated antennae, their length and the nature of the polymer all modulate the release of AmB. We find that the release of AmB from microsphere can be controlled and that the toxicity of this polyene can be reduced by controlling or directing a specific microsphere-target cell interaction.

Important fungal pathogens such as *Candida* sp. are responsible for hospital-acquired infections. The yeasts *Candida* express a number of adhesins capable of interacting with a variety of ligands including proteins and carbohydrates. The adhesin responsible of the adherence of *C. glabrata* to human epithelial cells is a glucan-cross-linked cell wall protein and binds to host cell carbohydrates, specially recognising asialo-lactosyl-containing carbohydrates.[26] Also, the use of other sugar 'tags' on the microspheres could be required for successful amphotericin B delivery and such data can be extended to several other drugs whose delivery to specific cells is required to reduce toxicity and provide better cell targetting. Depending on the desired mode of administration, the size of the carriers should be optimised, and the goal was to design nanoparticles drug system with a controlled delivery based on the biodegradable polymers: poly (L-lactic acid) and poly (L-lactic-co-glycolic acid).

Experimental

Materials

L-PLA ($\overline{M_w}=73{,}000$) and PLGA ($\overline{M_w}=34{,}000$) were purchased from Phusis (St. Ismier, France). L-PLA-C4-Gal **1a**, L-PLA-C6-Gal **1b**, L-PLA-C11-Gal **1c** and L-PLGA-C4-Gal **2a**, PLGA-C6-Gal **2b**, PLGA-C11-Gal **2c** were synthesised as described by Kassab et al.[21] The terminal L-PLA or PLGA carboxylic group was modified by coupling to a βamido-D-galactopyranoside possessing spacer chain of different lengths [C4 ($m=1$), C6 ($m=3$), C11 ($m=8$) Fig. 1] using the standard peptide conditions.

All solvents were distilled from CaH_2 in an inert atmosphere. AmB was obtained from Sigma; methylene chloride and Tween 80 (sorbitane ester polyoxyethylene) were purchased from Aldrich (France).

Molecular weight measurement

The molecular weights of derivatives **1a**, **1b**, **1c**, **2a**, **2b**, **2c** were measured by gel permeation chromatography (GPC) in a Waters chromatograph system equipped with triple columns using a differential refractometer for detection. Tetrahydrofuran (THF) was used as solvent. The number-average molecular weight ($\overline{M_n}$), the weight-average molecular weight ($\overline{M_w}$) and the polydispersity ($I=\overline{M_w}/\overline{M_n}$) of the polymers were calibrated using monodisperse polystyrene standards.

Preparation of AmB loaded microspheres

Microspheres were prepared by o/w emulsion solvent evaporation.[23] Briefly, 40 g Tween 80 were dissolved in 250 mL distilled water (w). 500 mg of galactosyl functionalised L-PLA (**1a**, **1b**, **1c**) or PLGA (**2a**, **2b**, **2c**) and AmB (10, 20, 30, 40, 60 or 80 mg) in 14 mL dichloromethane and 6 mL MeOH (o) were added to this aqueous phase. MeOH was necessary to dissolve the AmB in the organic phase. The mixture was stirred at 700 rpm using a Bioblock Scientific apparatus to form an o/w emulsion at room temperature and stirring continued for at least 12 h to allow solvent evaporation and microsphere formation. The AmB loaded microspheres were isolated by filtration, washed with distilled water and MeOH and freeze-dried.

Characterisation of microspheres

The diameter of L-PLA and PLGA microspheres was determined using a Coulter Laser 230 granulometer (Coultronics, Margency, France). The apparatus determines the mean and the standard deviation of each population. All samples were diluted in water and stirred automatically. The size of the microspheres was examined under a light microscope (Axioscope 50, Zeiss) and photographed with a Zeiss MC 80 Camera. The microporous structure of the microspheres was observed using an atomic force microscope (AFM). AFM images were obtained using a Topometrix Explorer AFM system. Scans were obtained by contact mode, in air with a scan rate of 2 Hz. A 150 μ scanner was used, equipped with 200 μ triangular cantilevers. Image resolution was 300 by 300 pixels. Images were unfiltered.

Determination of the microsphere AmB

The encapsulation efficiency was determined by UV spectrophotometry at $\lambda=409$ nm using a calibration curve [Shimadzu apparatus UV-2401 (PC)]. Briefly, microspheres (7 mg) loaded with AmB were dissolved in 7 mL dichloromethane and 3 mL MeOH. The amount of AmB trapped in microspheres was determined from the ratio: Encapsulated AmB/Introduced AmB×100.

Stability of the microspheres

The stability of the AmB loaded microspheres was tested by magnetic stirring in control culture medium as yeast culture conditions during 4 days, and their morphology was controlled by microscopic observations.

Micro-organisms and culture condition

The yeast strains used were *K. bulgaricus* (ATCC96631) and *S. cerevisiae* [obtained from 'Laboratoire de Microbiologie et Génétique ENSAM' at Montpellier (France)]. *K. bulgaricus* contains galactose-specific lectins and *S. cerevisiae* contains a mannose-specific lectin at the periphery. The yeasts were cultured aerobically at 25 °C in 100 mL Erlenmeyer flasks containing 20 mL liquid culture medium [4% glucose, 0.4% bactopeptone (Sigma), 0.1% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$; 0.02% $CaCl_2{\cdot}2H_2O$] with magnetic stirring.

Cell recognition

The L-PLA-Gal and PLGA-Gal microspheres (10 mg) were suspended in 20 mL Helm's acetate buffer (150 mM CH_3COONa, 3.75 mM $CaCl_2$ and 3 mM NaN_3), pH 4.5. An aliquot (10 μL) of the suspension was mixed with 10 μL yeast suspension (2×10^7 cells/mL) for 2 min. The mixture was then examined under a light microscope (Axioscope 50, Zeiss).

In vitro release and antifungal effect experiments

Microspheres (10 mg) were suspended in 20 mL culture medium in 100 mL Erlenmeyer flasks and inoculated with the yeasts. All the suspensions were stirred and incubated at 25 °C for at least 80 h. The growth of the yeasts was measured from the absorbance of the culture at 620 nm (0, 20, 40, 60 and 80 h) with a Perkin-Elmer (type: Coleman-295) spectro-photometer.

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