ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
INDUSTRIAL DE BARCELONA

UNIVERSIDAD POLITÉCNICA DE CATALUÑA

"Synthesis, characterization and biomedical applications of microbial polymalic and polyglutamic acids derivatives."

Presentado por: José Antonio Portilla Arias

Trabajo realizado bajo la dirección de los Drs.
Sebastián Muñoz Guerra y Montserrat García Álvarez

Barcelona, Febrero 2008

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4

Synthesis, Degradability and Drug Releasing Properties of Methyl Esters of Fungal Poly(β,L-malic acid)

Summary

Methyl esters of microbial poly(β,L-malic acid) for conversion degrees of 25, 50, 75 and 100% were prepared by treatment of the polyacid with diazomethane. Esterification proceeded with retention of the molecular weight of the parent polyacid and the copolymers displayed a blocky microstructure consisting of short segments of malic and methyl malate sequences. The thermal stability of the copolyesters was lower than those of the parent homopolymers and all of them were fairly crystalline with melting temperatures within the 170-175 ºC range.

They were degraded rapidly by water, the hydrolysis rate being highly depending on the methylation degree. Microspheres with mean-average diameters in the 1-20 μm range were prepared from the 100% methylated product by the emulsion-evaporation solvent method. Encapsulation of erythromycin was efficiently performed in these microparticles and its releasing upon incubation in simulated physiological medium was evaluated for different drug loads. Drug delivery was observed to occur by a releasing mechanism largely determined by the hydrodegradation of the host polymer and independent on the amount of loaded drug.

4.1. Introduction

Poly(malic acid) (PMLA) is a carboxylic-functionalized polyester that can be produced by either chemical synthesis or biological fermentation of myxomycetes and certain filamentous fungi[1]. Both α- and β-structures, either racemic or optically pure, may be obtained by chemical methods whereas microorganisms exclusively generate PMLA of extremely high optical purity. Since production costs by both routes are fairly high, PMLA is a hardly accessible product whose properties and applications have not been extensively investigated so far. Nevertheless, PMLA displays exceptionally good features for being used as drug carrier. It is a perfectly biodegradable and biocompatible polymer that is metabolized in the mammalian tricarboxylic acid cycle.[2]

Some PMLA derivatives have been proposed for drug targeting[3] as components of crosslinked prodrugs[4], and also as scaffolding for tissue regeneration.[5] Recently, a nanoconjugate prototype of drug delivery system based on PMLA produced by *Physarum polycephalum* has been reported for brain cancer chemotherapy.[6] PMLA is readily water-soluble and is hydrolyzed very fast in aqueous environment.[7]

Blocking of carboxylic side groups of PMLA has been a strategy frequently used to modify its properties and simultaneously to slow down its hydrolysis under the assumption that the convenient biological behaviour of the parent polyacid is retained. A good amount of work has been made on the benzyl[8] and methyl[9] esters of chemically synthesized PMLA. Furthermore, coupling of PMLA with alkyltrimethylammonium surfactants bearing long alkyl chains has been shown to be a reliable method to produce stoichiometric ionic complexes with a biphasic amphiphilic nanostructure[10] able to lodge hydrophobic drugs in the paraffinic subphase .[11]

So far, little has been reported on the biodegradability of PMLA derivatives other than the chemically prepared benzyl and hexyl esters. According to Holler *et al.*[12] naturally-occurring PMLA can be degraded by the hydrolase of *Physarum polycephalum,* an exohydrolytic enzyme that degrades specifically L-units. The non-enzymatic hydrolysis of synthetic PMLA was extensively studied by Braud and Vert.[7]

Regardless chirality, degradation proceeds rapidly according to an auto-catalytic random scission of the main-chain ester bonds to yield malic acid as the final degradation product. The hydrolytic degradation of PMLA benzyl esters appeared much more complex. In contrast to PMLA, the fully benzylated product is resistant to hydrolysis whereas partially converted PMLA degrades depending on the esterification degree because of faster degradation of acid rich segments.

It has been also shown that pellets made from these copolymers, as well as large devices made of poly(lactic acid) degraded heterogeneously and much faster than corresponding films and particles because of acidic autocatalysis and diffusion-dependent phenomena.[13] Barbosa *et al.*[14] have recently reported on the water degradation of nanoparticles made of benzyl and hexyl esters of PMLA[15] and have observed that hydrolysis is not greatly influenced by the esterification degree. These authors found that nanospheres degraded with a progressive decrease in the molecular weight of the copolymers demonstrating that the degradation proceeded by a random hydrolytic cleavage of the main chain ester bond.

In this work we wish to report on the synthesis, hydrolytic degradation and drug delivering properties of methyl esters of biosynthetic PMLA. Methylation with diazomethane of fungal PMLA has proven to be an effective method to produce poly(α-methyl β,L-malic acid) (PMLA-Me) without perceivable breaking of the initial polymer chain.[9b]

The method is applied here to prepare a set of partially methylated products with different conversion degrees, which will be designated in this work as *co*PMLA-(Me_xH_y), where x and y refer to the percentages of methyl malate and malic units contained in the copolymer. The hydrolytic degradation of these products is comparatively evaluated and the release of erythromycin from microspheres made of PMLA-Me is quantitatively examined. To our knowledge it is the first time that methyl esters of polymalic acid are investigated in this regard.

4.2. Experimental

4.2.1. Materials. Poly(β,L-malic acid) of microbial origin was used in this work. It was obtained by cultivation of *Physarum polycephalum* and subsequent purification as described in detail elsewhere.[12] The final polyacid was NMR pure and had a M_w = 34,300 Da and M_w/M_n =1.08, as determined by GPC. Erythromycin, assay 95% (NT), was supplied by Fluka and lipase from *Candida cylindracea* (EC 3.1.1.3, Type VII, 943 units mg^{-1}) was purchased from Sigma. Organic solvents were analytical grade and used without further purification. Water used for buffers preparation was doubled distilled and deionized in a "Milli-Q" system.

4.2.2. Esterification. A solution of diazomethane[9] in ether (0.25 M) was added in different ratios, according to the methylation degree to be attained, to a solution of PMLA in dry acetone (500 mg, 4.3 mmol) and the mixture left under stirring at room temperature for 1 h. In all cases, the reaction proceeded with precipitation of the partially esterified PMLA.

The reaction mixture was then evaporated under vacuum and the residue was dissolved in a small amount of *N*-methyl pyrrolidone (NMP) and added with cold diethyl ether, the PMLA methyl esters being recovered by filtration as white powders. *co*PMLA-(Me_xH_y) with nominally methylation degrees of 25, 50 and 75% in addition to fully methylated PMLA-Me were prepared by this method.

4.2.3. Hydrolytic and enzymatic degradation. In the case of *co*PMLA-($Me_{25}H_{75}$) and *co*PMLA-($Me_{50}H_{50}$), the degradation study was performed using a solution with a concentration of 1 mg of copolymer in 1 mL of buffered saline solutions of selected pH's (pH 4.0 citrate buffer, pH 11.0 Na_2HPO_4/NaOH buffer, and pH 7.4 phosphate buffer).

Degradation of the non water-soluble *co*PMLA-($Me_{75}H_{25}$) and PMLA-Me compounds was performed on 5 mm-diameter and 1 mm-thick disks, which were cut from polymer films prepared by casting from hexafluoroisopropanol solutions (10 $mg \cdot mL^{-1}$). Disks were placed into vials and covered with 5 mL of buffered solutions. The sample vials were sealed to avoid evaporation of the fluids and stored at 37 °C in a heat chamber.

The enzymatic degradation was carried out with lipase (0.1 $mg \cdot mL^{-1}$) in phosphate buffered saline solution (pH 7.4, 37°C). At the end of the incubation period, the disks were withdrawn, from the incubation medium, washed with distilled water, dried, weighted and analyzed by gel permeation chromatography (GPC) by triplicate.

4.2.4. Preparation of microspheres, hydrodegradation and erythromycin release. Microspheres were prepared by the emulsion-evaporation solvent method. PMLA-Me was dissolved in chloroform (4 $mg \cdot mL^{-1}$), and then erythromycin was dissolved in the polymer solution at concentrations of 10, 20 and 30 % (w/w) respect to PMLA-Me. The solution was added with different amounts of poly(vinyl alcohol) (4, 6, 8 and 10 %), which acts as emulsifier, and stirred at different speeds (400, 800, 1200 and 1600 rpm) at room temperature until the solvent evaporated completely.

The evolution of microspheres formation was followed by optical microscopy. At the end of the process, the microspheres were rinsed three times with distilled water and finally recovered by freeze-drying.

The hydrolytic degradation of microspheres was performed by placing 10 mg of the particles into vials and adding 5 mL of buffered solutions at different pH's 4.0, 11.0 and 7.4.

The sample vials were sealed and stored at 37 °C in a heat chamber.

The temperature effect was evaluated at pH 7.4 in samples stored at 37, 60, and 80 ºC.

The enzymatic degradation was carried out in a phosphate buffered saline solution (pH 7.4, 37°C) with lipase (0.1 $mg \cdot mL^{-1}$). At predetermined intervals, the microparticles were withdrawn, washed with distilled water, filtered and analyzed by GPC. The analysis was carried out by triplicate.

The amount of active ingredient incorporated into the microspheres was estimated by UV-vis spectrophotometry. Randomly selected loaded microspheres (10 mg) with drug loads of 10, 20 and 30% w/w were dissolved in $CHCl_3$ and the erythromycin present determined using the appropriate blank and calibration curve.

The *in vitro* release test was carried out by incubation of the 10 mg of loaded microspheres in 10 mL the phosphate saline buffer (pH 7.4 at 37 ºC. Aliquots of 2 mL were taken from the releasing media at scheduled times and the removed volume being replaced by fresh medium every time. The amount of delivered drug was estimated by spectrophotometry and the cumulative drug concentration plotted against release time.

4.2.5. Measurements. ^{1}H and ^{13}C NMR spectra were recorded on either a Bruker AMX-500 NMR or a Bruker AC-250 instruments with samples dissolved in $CDCl_3$, DMSO or D_2O and using TMS as reference.

Gel permeation chromatography was performed using a Waters equipment provided with IR an UV detectors. Non water soluble PMLA-Me and *co*PMLA-($Me_{75}H_{25}$) were chromatographied using 0.005M sodium trifluoroacetate-hexafluoroisopropanol (NaTFA-HFIP) and chromatograms were calibrated against PMMA. Chromatography of PMLA, *co*PMLA-($Me_{25}H_{75}$) and *co*PMLA-($Me_{50}H_{50}$) was carried out in phosphate buffer pH 7.0 and chromatograms were calibrated against poly(ethylene oxide) (PEO).

Optical microscopy was carried out in an Olympus BX51 microscope equipped with a digital camera system. Scanning electron microscopy (SEM) was used to examine the morphology of samples before and after degradation. Gold coating was accomplished by using a Balzers SDC-004 Sputter Coater. The SEM microphotographs were taken with a JEOL SSM-6400 instrument.

Calorimetric measurements were performed with a Perkin-Elmer Pyris DSC instrument calibrated with indium. Sample weights of about 3-6 mg were used at heating and cooling rates of 10 $°C \cdot min^{-1}$ under a nitrogen atmosphere.

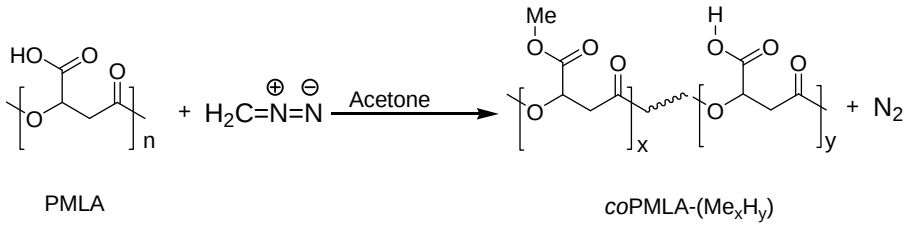
Thermogravimetry (TGA) experiments were carried out in a thermobalance Perkin-Elmer TGA6 under a circulating nitrogen flow at a heating rate of 10 ºC·min^{-1}. Samples weights of about 15 mg were used in all cases.

X-ray diffractograms were obtained from powdered samples in a Debye-Scherrer diffractometer INEL CPS-120, using Cu Kα radiation of wavelength 0.1541 nm. The size distribution of the PMLA-Me microparticles was measured by laser dispersion/PIDS using a Beckman Coulter LS13320.

Contact angles between water and solid surfaces were measured by means of an OCA 15+ Contact Angle measuring system supported by an SCA20 software (Dataphysics, Germany). Angle values were registered after 30 seconds of dropping the water onto the polymer surface and at least 10 measurements were made.

4.3. Results and discussion

4.3.1. Partially methylated poly(β,L-malic acid)s. Synthesis and characterization. The methylation procedure based on the treatment of PMLA with diazomethane in acetone (Scheme I) had been previously used by us for the efficient preparation of fully methylated polymalic acid, PMLA-Me. It has been applied now to the preparation of the partially esterified polymalic acids with nominal methylation percentages of 25, 50 and 75%, which refer to the diazomethane/polyacid ratios used for the reaction.



Scheme I. Esterification of PMLA with diazomethane.

Esterification results and characteristics of the resulting copolyesters together with similar data for PMLA and PMLA-Me are shown in Table 1.

Table 1. Esterification results and characteristics of polymers.

Polymer	Esterification		GPC[a]		Solubility[b]				CA[c]
	[Me] (%)	Yield (%)	Mw	PD	H_2O	Acetone	$CHCl_3$	HFIP	deg
PMLA	0	-	34 300	1.08	x	x	-	-	12
*co*PMLA-($Me_{25}H_{75}$)	20.2	97	32 600	1.25	x	-	-	x	20
*co*PMLA-($Me_{50}H_{50}$)	46.5	92	33 100	1.36	x	-	-	x	29
*co*PMLA-($Me_{75}H_{25}$)	75.1	97	34 200	1.28	-	-	-	x	40
PMLA-Me	100	93	34 100	1.44	-	-	x	x	49

[a] Weight-average molecular weight (Mw) and polydispersity (PD) measured by GPC. [b]All polymer are soluble in DMSO, NMP and pyridine. [c] Contact angles measured on films made by casting from DMSO.

The ^{1}H-NMR analysis revealed that the conversion actually attained in the copolyesters was 20.2, 46.5, and 75.1, which are pretty close to the nominal values. Yields were higher than 90% for all the cases. The molecular weights of these copolyesters were practically coincident to the molecular weight of the parent polyacid indicating that very light degradation took place upon esterification.

Consequently, polydispersities were found to increase slightly but remaining below 1.5 for the copolymers as well as for the fully methylated polymer.

The microstructure of the copolyester was investigated by ^{13}C-NMR taking benefit from the sensitivity to dyad sequence displayed by the main chain carboxylic signal. As it is shown in Figure 1, this signal appears split into three peaks which could be assigned to the four types of dyads, MM, HH, MH and HM that are feasible for the copolyester chain made of malic (H) and methyl malate (M) units. Comparison between the homopolymers and copolymer spectra leads to assign unequivocally the peaks appearing at 168.20 and 168.25 ppm to homodyads HH and MM, respectively. Consequently, the peak at 168.30 ppm must be attributed to heterodyads MH and HM. It should be noticed that such assignment is contrary to expectations since the peak arising from heterodyads is usually located intermediate between homodyads peaks.

Although we have presently not definite explanation for this assignment, magnetically environment effects due to specific intramolecular interactions between neighboring units can be invoked to be behind this striking result.

Dyad contents were estimated from peak areas and used to calculate the sequence lengths as well as the randomness degree, *R*, present in the copolymers.

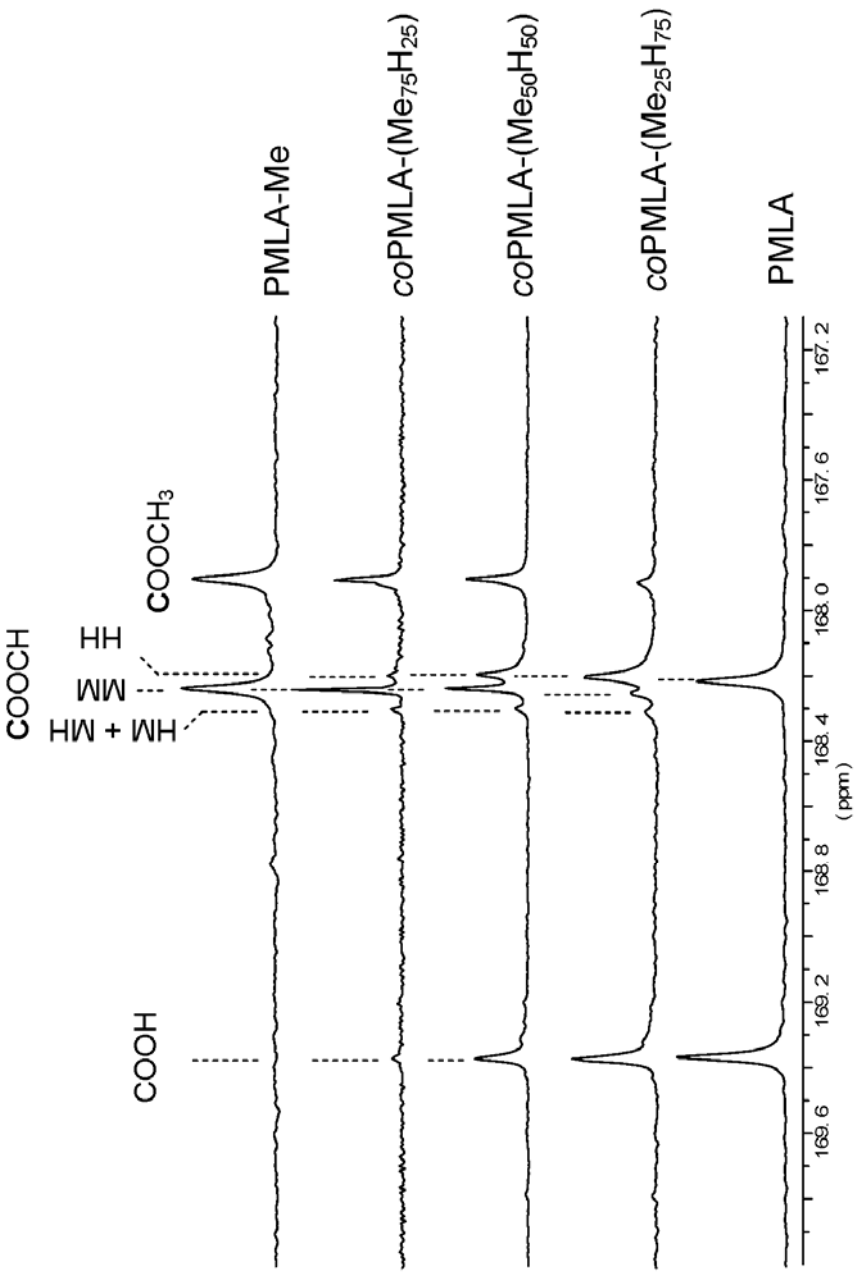


Figure 1. ^{13}C NMR spectra of PMLA, PMLA-Me and copolymers in the carbonyl region.

The *R* resulting values indicate that the microstructure of copolyesters is far from statistical, the carboxylic and methylcarboxylate units being grouped in blocks of five- to twenty-units repeating length depending on the global composition of the copolymer. This result is consistent with the fact that the partially esterified PMLA precipitated from the reaction medium changing the accessibility of the hydroxyl groups to diazomethane as the reaction proceeds.

Table 2. Composition, number-average sequence lengths, and randomness of methylated PMLA.

	Composition (mole-%)[a]		Dyads[b] (mole-%)			Number-Average Sequence Length[b]		
Polymer	X_M	X_H	MM	MH+HM	HH	n_M	n_H	R^b
PMLA	0	100	-	-	100	-	274	0.0
*co*PMLA-($Me_{25}H_{75}$)	20.2	79.8	18.6	13.0	68.4	3.9	11.5	0.3
*co*PMLA-($Me_{50}H_{50}$)	46.5	53.5	41.2	7.6	51.2	11.8	14.5	0.2
*co*PMLA-($Me_{75}H_{25}$)	75.1	24.9	74.0	9.0	17.0	17.4	4.8	0.3
PMLA-Me	100	0	100	-	-	182		0.0

[a] Determined from the signals arising from main chain methyne protons.
[b] Values estimated by deconvolution of main chain carbonyl carbon peaks and calculations made according to ref. 16.

The solubility of methylated polymalic acid appeared to be strongly dependent on the esterification degree. As shown in Table 1, the polyacid PMLA is soluble in water and acetone whereas PMLA-Me is soluble in $CHCl_3$. The solubility of *co*PMLA-($Me_{50}H_{50}$) and *co*PMLA-($Me_{25}H_{75}$) in pure water is limited but a complete solubilization of the copolymer is reached by increasing the ionic strength. The *co*PMLA-($Me_{75}H_{25}$) is non-water soluble, and none of the partially methylated products are soluble in either acetone or $CHCl_3$.

Static contact angles measured on films were consistent with the hydrophilic behavior expected for *co*PMLA-(Me_xH_y), the angle becoming steadily wider as the degree of methylation increases.

The thermal stability of *co*PMLA-(Me_xH_y) was evaluated by thermogravimetry under inert atmosphere. TGA traces for copolymers and homopolymers are compared in Figure 2a and characteristic decomposition temperatures and remaining weights determined in these experiments are compared in Table 3.

Both onset and maximum rate decomposition temperatures are lower for the partially methylated products than for either of the two homopolymers. We reported recently on the thermal degradation of PMLA and PMLA-Me[17] showing that different decomposition mechanisms operate in each case.

Decomposition of the polyacid takes place through an unzipping mechanism with release of fumaric acid whereas the polymalate degrades by a random scission process leading to oligomeric fragments. According to the constitution of *co*PMLA-(Me_xH_y) copolymers, a complex process involving the two depolymerization mechanisms should be expected to occur in the thermal decomposition of these copolymers.

Table 3. Thermal Behaviour of polymers.

	DSC[a]			TGA[b]		
Polymer	T_m (ºC)	ΔH_m ($J \cdot g^{-1}$)	T_g (ºC)	^oT_d (ºC)	^mT_d (ºC)	W (%)
PMLA	216	42	110	236	250	5
*co*PMLA-($Me_{25}H_{75}$)	174	72	n.o.	186	228	3
*co*PMLA-($Me_{50}H_{50}$)	172	58	n.o.	198	245	2
*co*PMLA-($Me_{75}H_{25}$)	171	41	45	202	250	1
PMLA-Me	148	42	41	211	251	1

[a]Melting (T_m) and glass transition (T_g) temperatures and melting enthalpy (ΔH_m) measured by DSC for samples coming directly from synthesis. [b]Onset decomposition temperature measured at 5% of loss of initial weight, (^oT_d), maximum rate decomposition temperature (^mT_d), and remaining weight (W).

The polyacid PMLA displays a much higher T_g than the methylated product PMLA-Me (110 against 41 ºC) indicating the highly restricting effect of the acid groups on molecular mobility. To determine T_g samples were heated above their respective melting temperatures and then rapidely cooled to -10 ºC.

Nevertheless, glass transitions were not clearly observable in copolymers *co*PMLA-(Me_xH_y) by DSC so that only the T_g of *co*PMLA-($Me_{75}H_{25}$) could be determined with acceptable confidence. The value obtained was 45 ºC which is consistent with the increasing trend that should be expected along the series for decreasing contents in methylated units. PMLA has been reported to show crystalline melting at 216 ºC provided that it is well dry[12b] but its crystal structure is not known.

On the other hand PMLA-Me is a high crystalline polymer able to crystallize from the melt displaying two crystal modifications depending on molecular weight and crystallization conditions.[9b] The DSC traces of copolymers *co*PMLA-(Me_xH_y) are shown in Figure 2b revealing the occurrence of melting in all them at temperatures around 170-175 ºC with associated enthalpies of fairly high values.

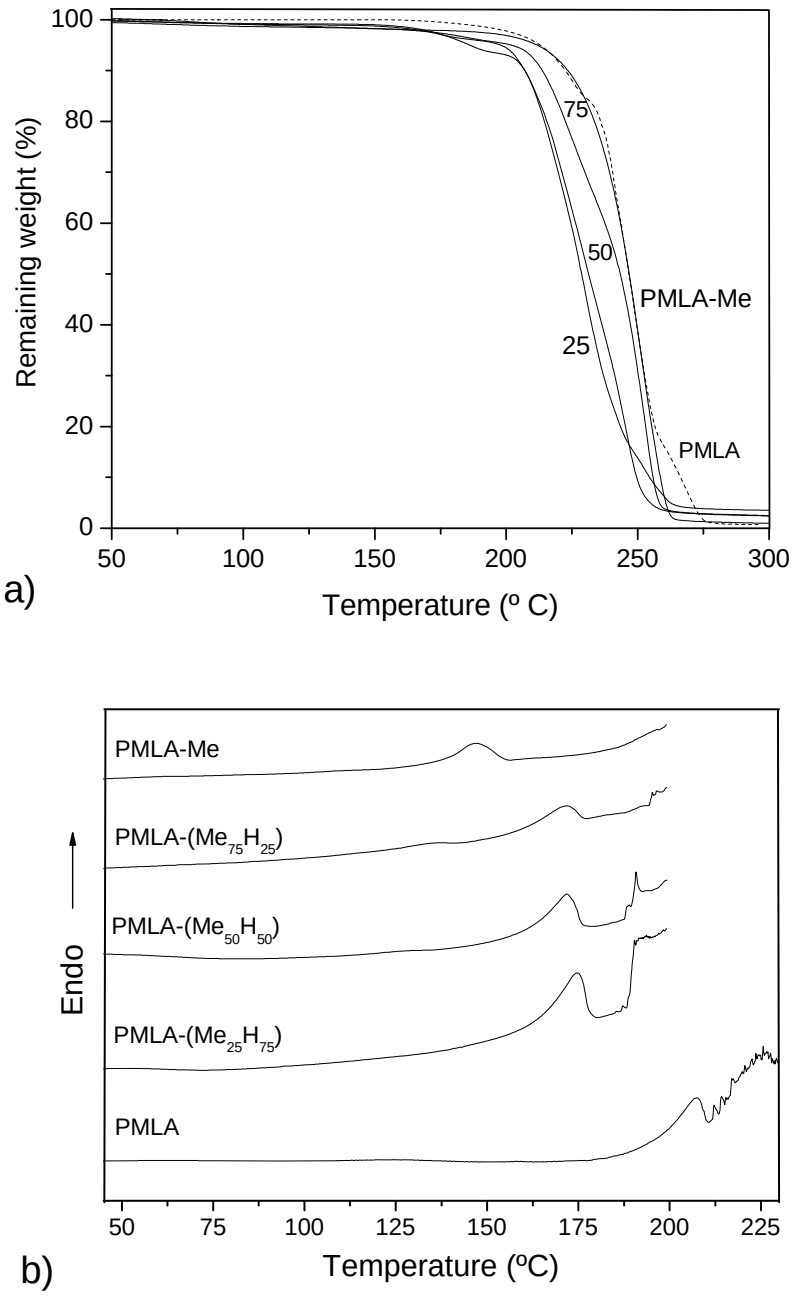


Figure 2. TGA (a) and DSC (b) traces of PMLA, PMLA-Me and copolymers. (percentage or methylated units indicated by labels).

Powder X-ray diffraction of these copolymers produced discrete scattering characteristic of semicrystalline material. The intensity profiles of the whole series and those of the parent homopolymers PMLA and PMLA-Me, are compared in Figure 3 where it can be seen the great structural similitude existing between PMLA-Me and *co*PMLA-($Me_{75}H_{25}$), both of them being highly crystalline compounds. The other two copolymers with lower esterification degrees display more complex diffraction patterns and they appear to be less crystalline.

A close comparison of the peak spacings appearing on the diffraction profiles indicate however that the structure of PMLA-Me seems to be retained in these compounds although accompanied by a second crystal structure reminiscent of that adopted by PMLA.

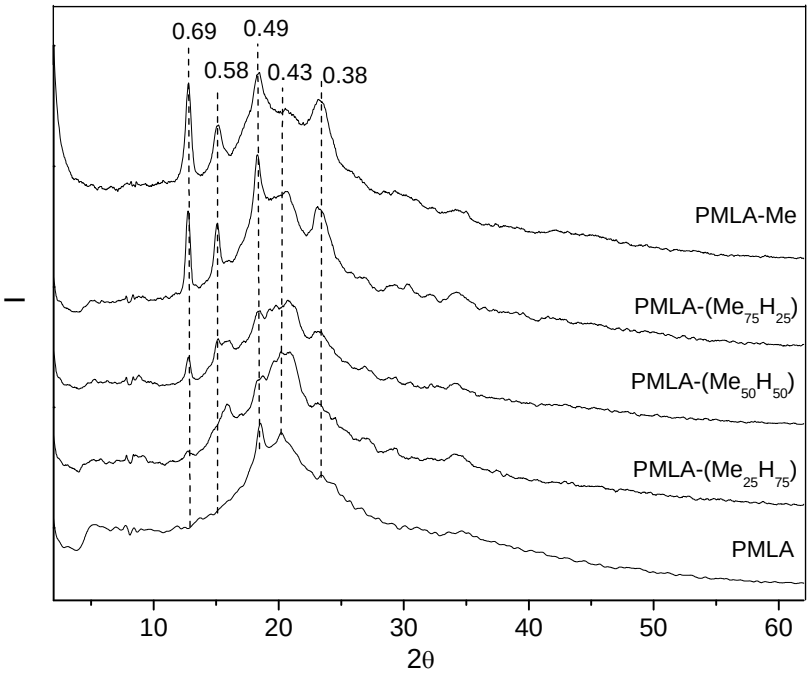
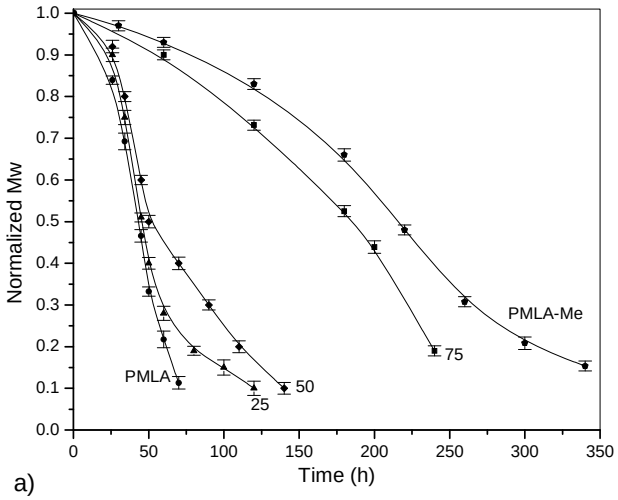


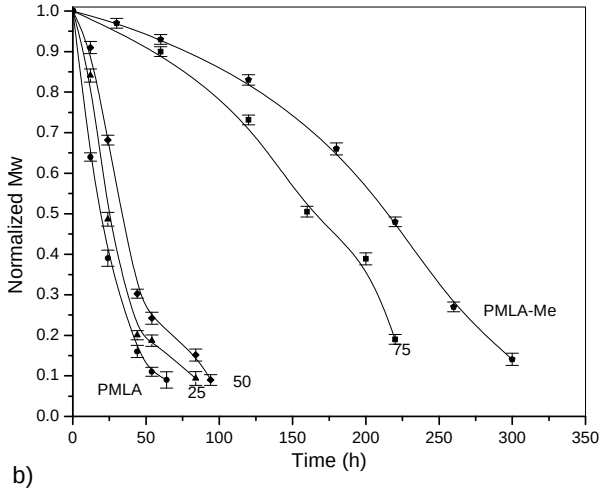
Figure 3. Intensity X-ray diffraction profiles of PMLA, PMLA-Me and copolymers. Most characteristic Bragg spacings labeled in nm.

4.3.2. Hydrolytic degradation. The hydrolytic degradation study of *co*PMLA-(Me_xH_y) was carried out under different conditions depending on their solubility in water. *co*PMLA-($Me_{25}H_{75}$) and *co*PMLA-($Me_{50}H_{50}$) were dissolved in aqueous buffer and incubated at 37 ºC at pH 7.4 with or without added lipase. Conversely, disks of the non-water soluble copolymer *co*PMLA-($Me_{75}H_{25}$) and the homopolymer PMLA-Me were incubated at pH 7.4 and at 37 ºC and 80 ºC to evaluate the effect of temperature.

Additional incubations at pH 4.0 and 11.0 were carried out at 37 ºC for all the polymers to evaluate the influence of the medium pH. Results afforded by this study are plotted in Figures 4 and 5. As expected, hydrolysis appears to be delayed by esterification, regardless the incubation conditions that are applied. A clear difference is observed between water soluble and non-water soluble products. Thus, PMLA incubated at pH 7.4 is almost completely degraded in 75 hours at 37 ºC whereas PMLA-Me requires a period nearly five times longer. Intermediate behaviors are observed for the copolymers, with degradation rates increasing with the methylation degree.



a)



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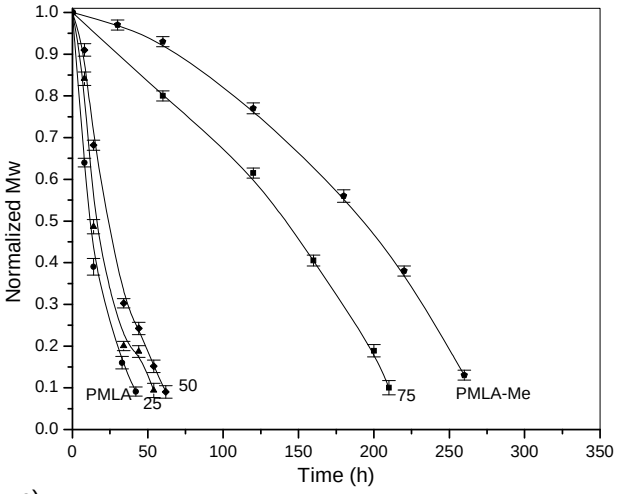

c)

Figure 4. Hydrolytic degradation at 37ºC and a) pH 7.4, b) pH 4.0, c) pH 11.0 (percentage or methylated units indicated by labels).

On the other hand, hydrolysis of water-soluble copolymers appeared to be significantly enhanced by enzymes. Approximately half time periods were required to attain comparable degradation levels in these copolymers when incubation was performed in the presence of lipase. The effect of temperature on degradation rate was also noticeable but rather striking; both PMLA-Me and *co*PMLA-($Me_{75}H_{25}$) degraded faster at 80 ºC than at 37 ºC, but the difference was not so large as it should be expected for incubations that carried out at temperatures above and below the T_g of the polymers.

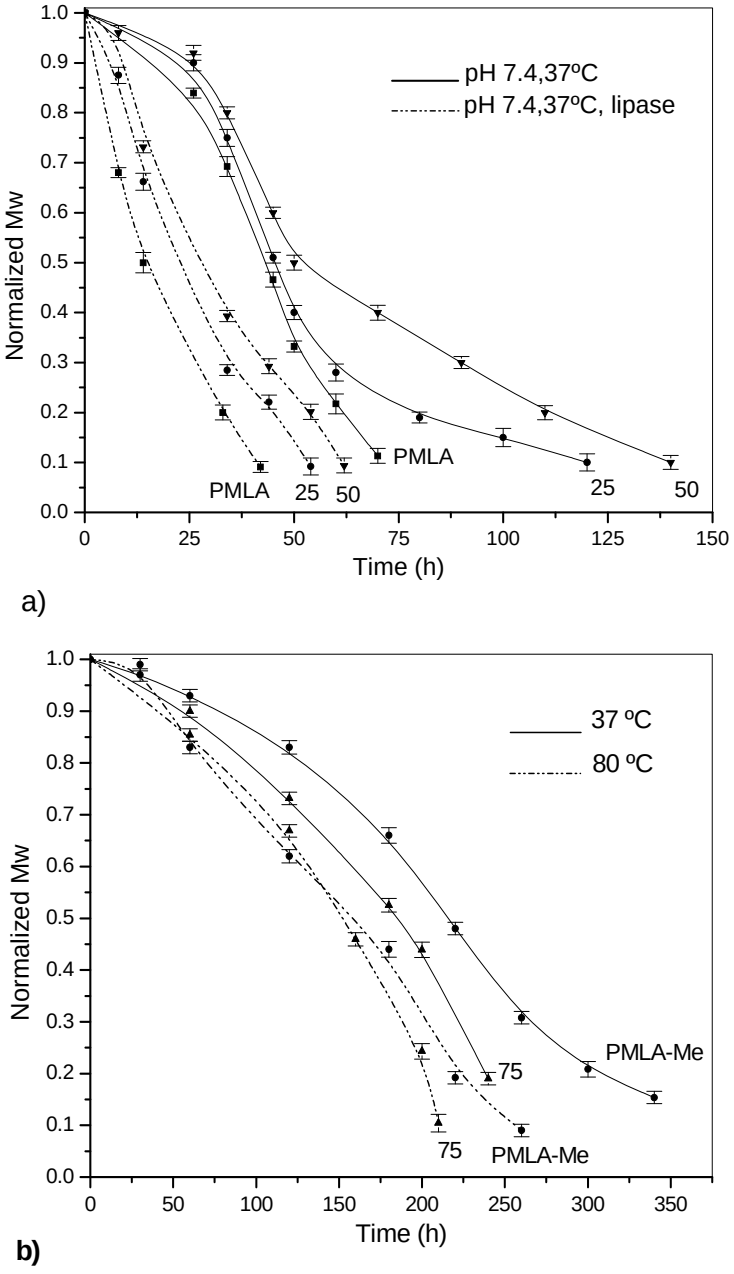

Figure 5. Plots illustrating the effect of enzyme on degradation of buffer-soluble copolymers (a) and temperature on degradation of insoluble copolymers. (percentage or methylated units indicated by labels).

The hydrolysis mechanism was investigated by ^{1}H-NMR analysis of the degradation products generated by PMLA-Me and *co*PMLA-($Me_{25}H_{75}$) upon incubation in pure water at room temperature. The NMR spectra recorded from the mother solution of PMLA-Me for increasing incubation times are compared in Figure 6. Initially large amounts of methanol were present in the aqueous solution whereas no other products were detectable.

The amount of methanol increased with time and later polymalic acid entered in the incubation medium whereas no signs of partially methylated PMLA were detected. At the end of the process, malic acid and methanol were the only compounds present in the incubating medium. Similar results were obtained in the hydrolysis of *co*PMLA-($Me_{25}H_{75}$).

All these results indicate that hydrolytic degradation of methyl esters of PMLA must take place by hydrolysis of the methyl carboxylate side group followed by hydrolysis of the carboxylate main chain.

According to the observations previously reported for the benzyl esters of synthetic PMLA, the carboxylic side groups initially liberated should enhance the random hydrolysis of the ester backbone groups, which otherwise would be highly resistant to breaking.[13]

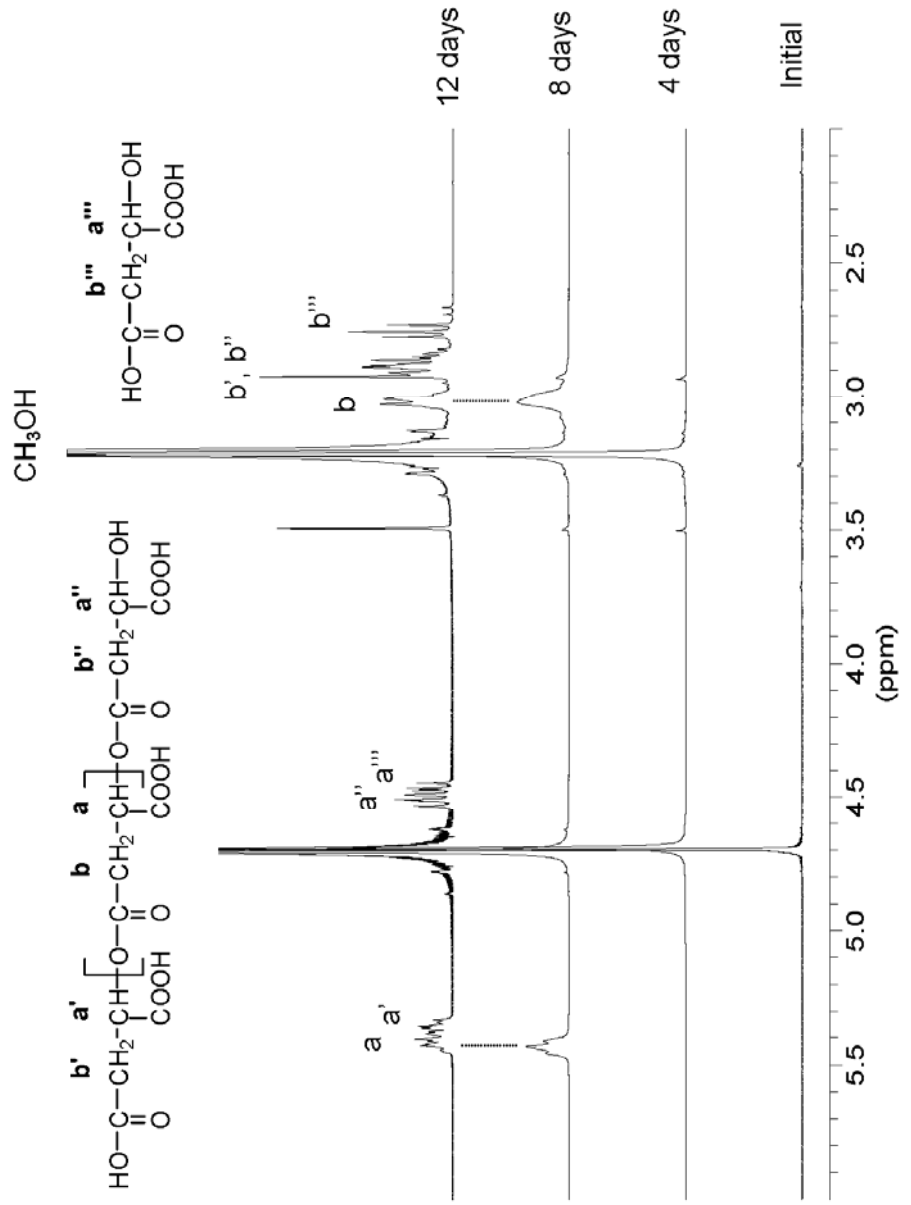

Figure 6. ^{1}H NMR spectra of the aqueous medium used for degrading PMLA-Me recorded at the indicated incubation times.

4.3.3. Microspheres of PMLA-Me: Preparation, hydrolytic degradation and erythromycin release. Microspheres of PMLA-Me were obtained by the emulsion-evaporation solvent method using chloroform as organic phase. The particle diameter could be adjusted by both stirring speed and amount of PVA added as emulsifier, their effects being illustrated in Figure 7.

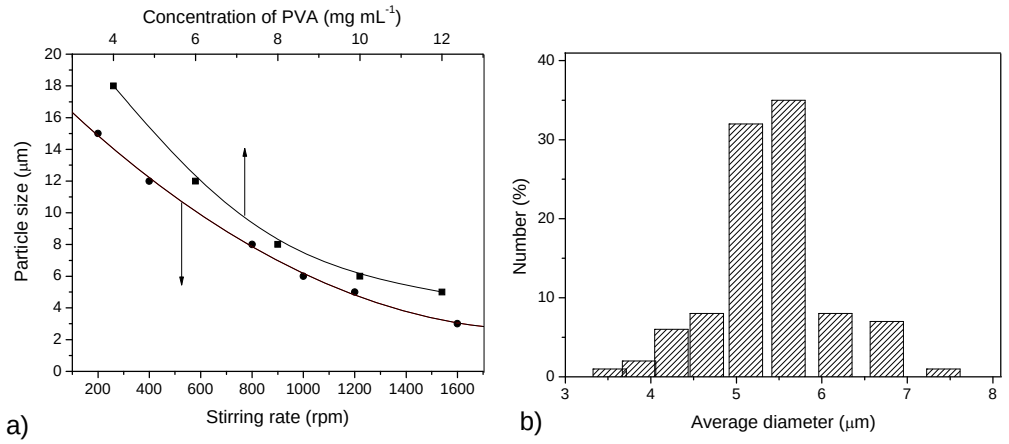

Figure 7. a) Influence of PVA concentration (at a stirring rate of 1000 rpm) and stirring rate (at a PVA concentration of 8 mg·mL^{-1}) on particle size and b) Particle distribution of microspheres selected to study.

Particle diameters between 1 and 20 μm with an approximate Gaussian distribution of sizes were feasible by independent adjusting of these two factors. The well spherical shape adopted by these particles is clearly revealed in the optical micrograph shown in Figure 8a, which was taken from the aqueous emulsion of average 5 μm-diameter microparticles before drying.

As it is seen in the SEM picture in Figure 8b, the spherical shape was preserved, after drying, without significant alteration, although the particle surface become rather rough and frequently holed. This 5-μm diameter particle population of microspheres was selected for conducting the degradation and drug releasing studies that are described below.

With the aim at providing a comprehensive account of the hydrodegradability of the PMLA-Me microspheres, they were incubated in aqueous medium and the effects of pH, temperature and lipase on hydrolysis rate were comparatively evaluated. Results are shown in Figure 9 where the molecular weight of PMLA-Me is plotted against incubation time for the different conditions assayed.

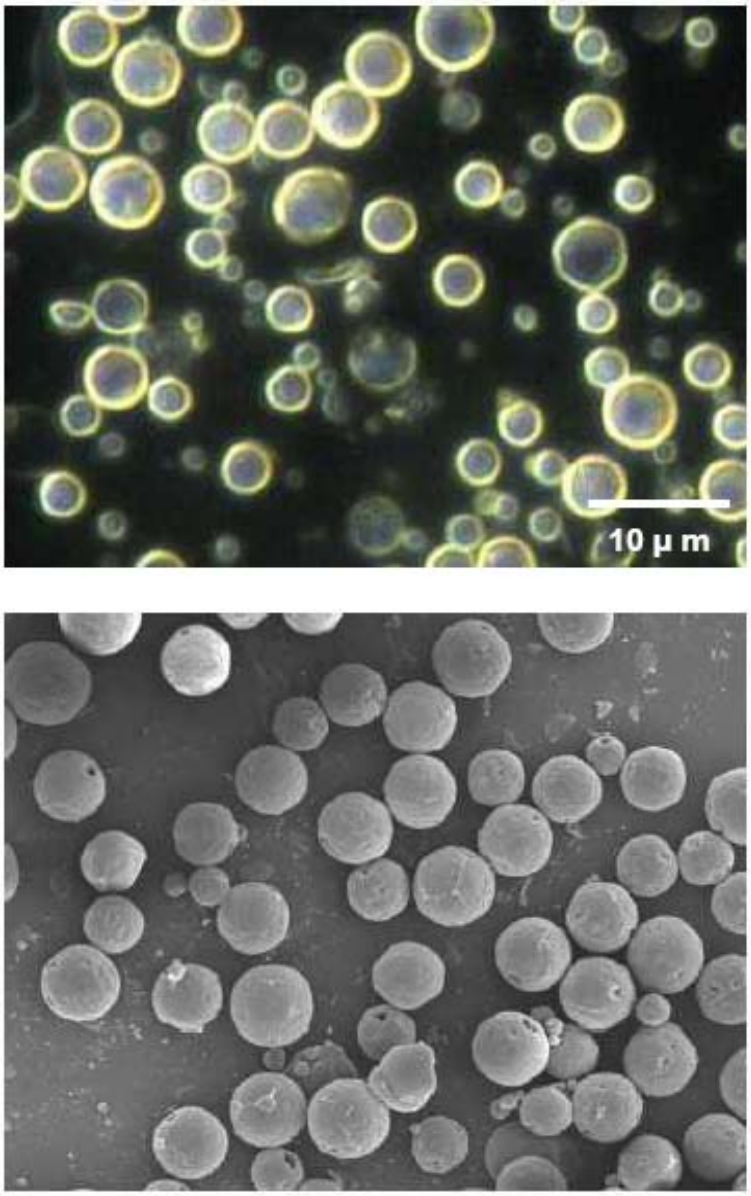

Figure 8. Microspheres of PMLA-Me. Top: Optical micrograph of microspheres suspended in water. Bottom: SEM micrograph of microspheres after freeze-drying (bottom).

Firstly, it should be noted that degradation of PMLA-Me in microspheres appears to be slower than in films. This is a rather striking result since a higher hydrodegradability should be expected for the particles given the more efficient polymer-water contact that is feasible in such case. The screening effect of some emulsifier adhered to the particle surface could be considered in this regard to explain this anomalous result.

On the other hand, the effect of pH and temperature is slight and operating in the expected sense. High hydrolysis rates are observed at higher temperatures and for both basic or acidic pH's. Also the enhancing effect of lipase is clearly perceived but not with the intensity observed for the water-soluble copolymers discussed above.

The morphological alteration underwent by the particles as consequence of the degradation process is illustrated in Figure 10 where the SEM pictures of the undegraded sample and the samples degraded down to molecular weights of 20% and 50% of the initial one are compared. According to the increasing roughness exhibited by the microspheres, an erosion mechanism entailing the particle surface seems to be operating. Nevertheless, most of particles appear severely crushed suggesting the concourse of degradation in bulk or a hollowed structure of the particle.

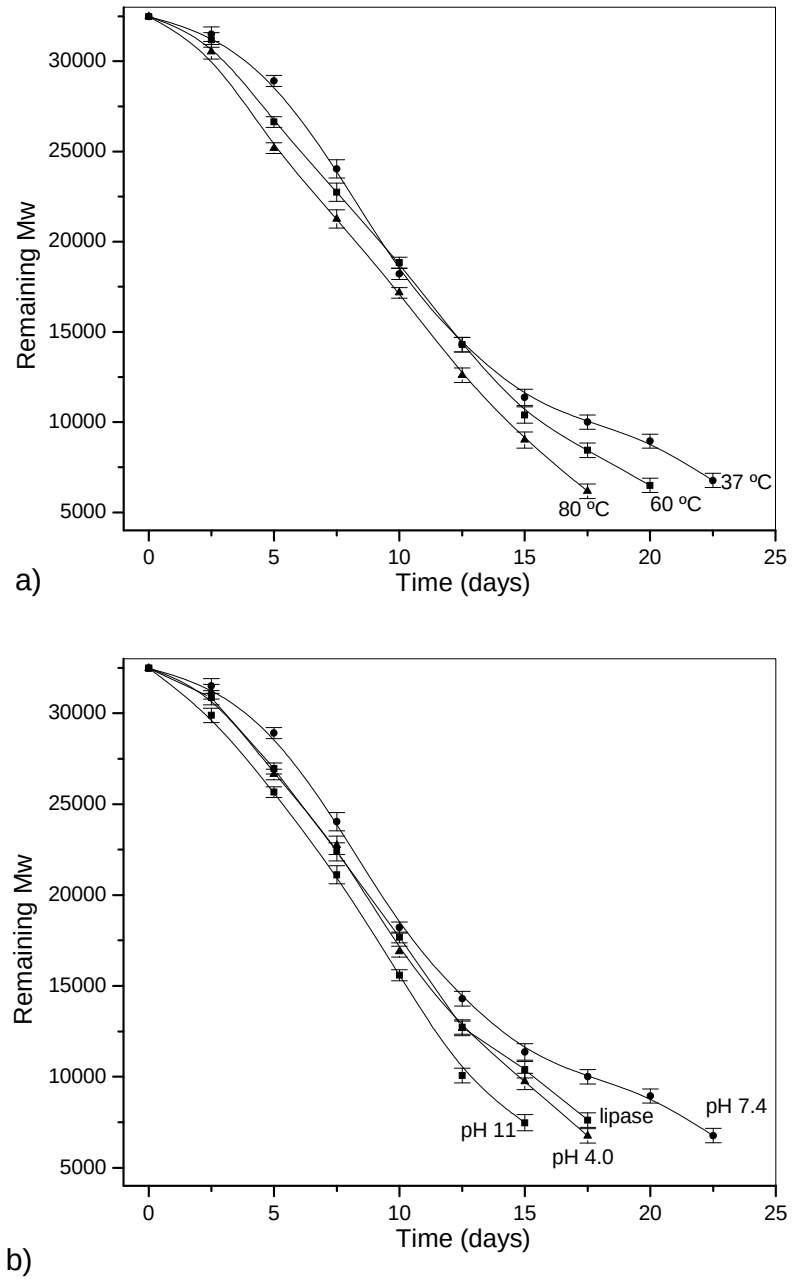

Figure 9. Compared mass loss of microspheres of PMLA-Me incubated at pH 7.4 and several temperatures (a) and at 37 ºC and several pH's (b).

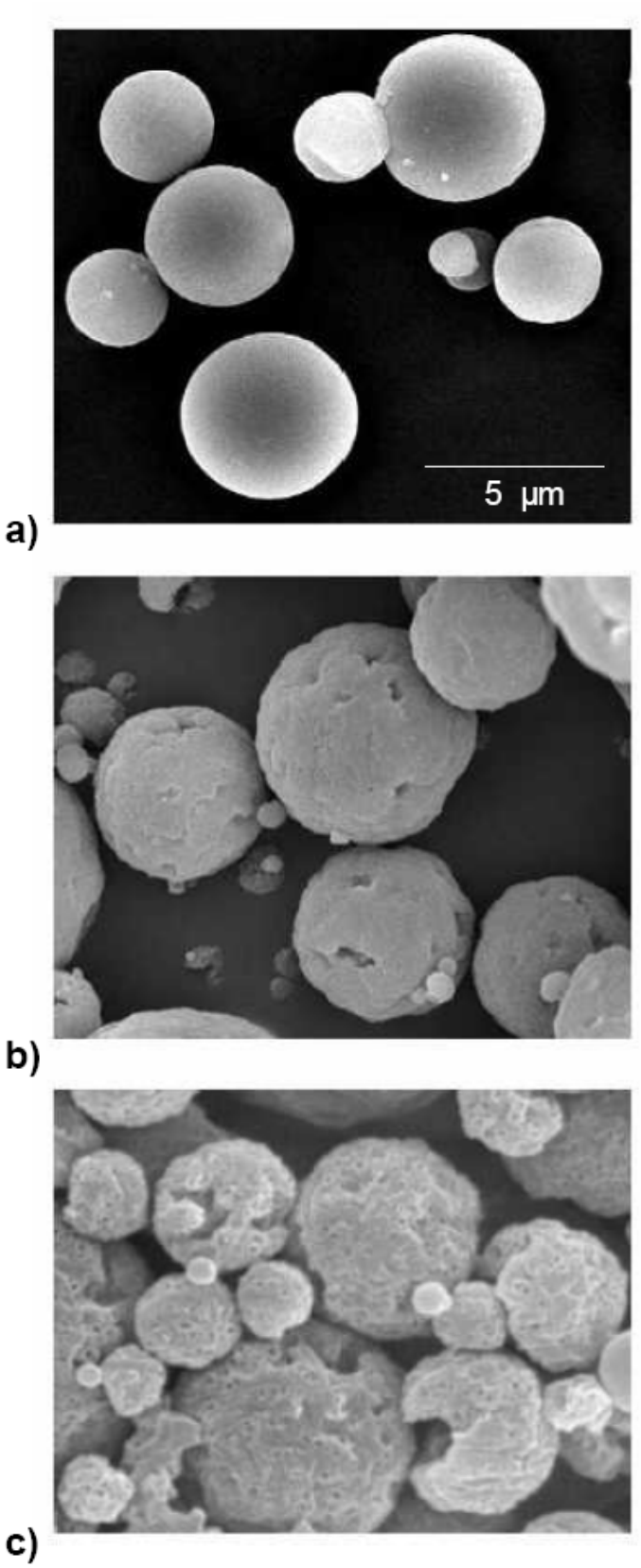

Figure 10. SEM micrographs of PMLA-Me microspheres subjected to hydrolysis. a) Initial, b) degraded down to 20% of initial Mw, and c) degraded down to 50% of initial Mw.

PMLA-Me microspheres loaded with 10, 20 and 30% of erythromycin were prepared and their drug delivering properties evaluated following the erythromycin release by UV spectroscopy. Encapsulation efficiencies were 84, 88 and 92% for loads of 10, 20 and 30% w/w of erythromycin respectively. DSC traces recorded from 30% erythromycin loaded and unloaded microspheres along with that of pure erythromycin are shown in Figure 11. Endothermic peaks characteristic of melting are seen at 147 ºC and 191 ºC for the unloaded polymer and antibiotic, respectively. In the loaded microspheres, a single melting peak at 138 ºC is observed indicating that the drug is homogeneously dispersed in the matrix of the polymer without phase separation.

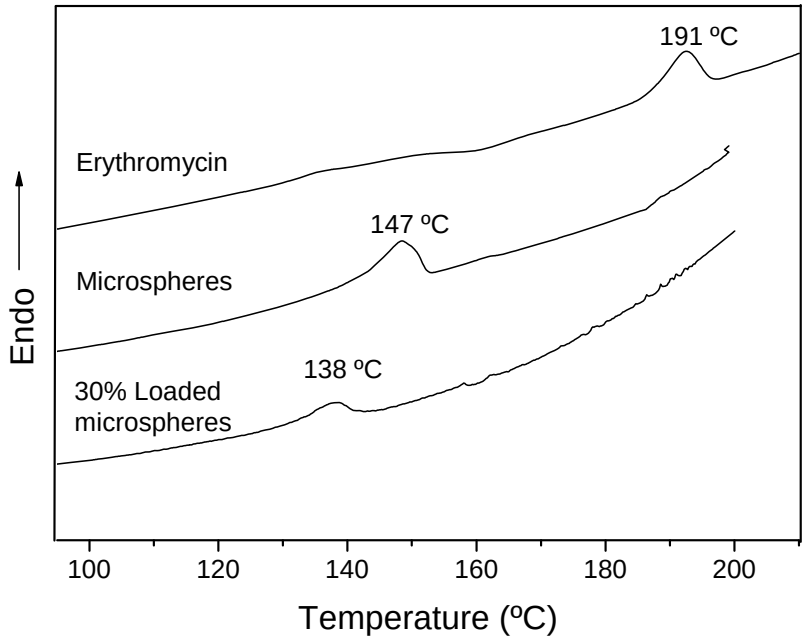

Figure 11. DSC traces of erythromycin loaded PMLA-Me microspheres and their components.

The releasing of erythromycin as a function of incubation time is plotted in Figure 12 for the three assayed loads. In all cases approximately one tenth of the loaded antibiotic is released after a few hours of incubation, which is interpreted as due to the detachment of compound that was adhered to the microspheres surface. The same releasing pattern is essentially shared by the three samples as far as drug is present in the microspheres, the fractional amount of erythromycin liberated at any given time being almost the same for all the assayed loads.

Accordingly, the complete delivery of the drug appears to occur at the same time for the three loads, which is approximately also the time needed for the total degradation of the microparticles.

Such a behavior leads to consider that the contribution of diffusion processes to the releasing mechanism is almost negligible. In other words, drug releasing seems to be exclusively determined by polymer degradation, otherwise the asymptotic behavior should be reached at shorter times for lower drug loads.

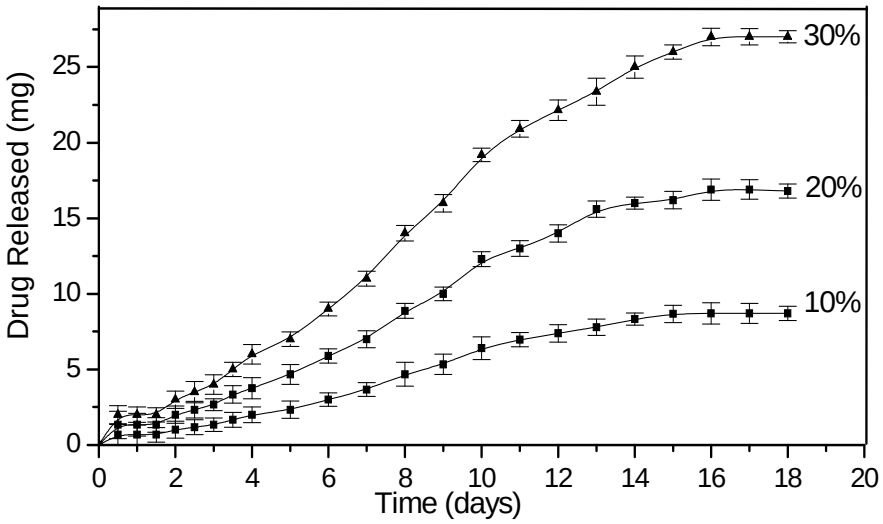

Figure 12. Erythromycin release profiles from PMLA-Me microspheres loaded with 10, 20 and 30% w/w of drug upon incubation in aqueous buffer at pH 7.4 and 37°C.

4.4. Conclusions

It is well known that esterification of poly(β,L-malic acid) is a more than difficult synthesis task due to the extreme chemical weakness of the main chain ester bond. Up to now all known copolymers based on PMLA were obtained by chemical synthesis starting from modified lactides. In this work we have succeeded in preparing the methyl esters of microbial PMLA for 25, 50 and 75 % of conversion by treatment of the polyacid with diazomethane without significant decreasing of the molecular weight.

The copolymers have a blocky microstructure consisting of short segments of malic and methyl malate sequences and their solubility is highly depending on composition. Both the fully methylated ester and the copolyesters are rapidly degraded by water through a mechanism that implies the hydrolysis of the methyl carboxylate side groups followed by the acidic self-catalyzed hydrolysis of the main chain ester bonds.

Microspheres with mean-average diameters in the 1-20 μm range were prepared from 100% methylated product by the emulsion-evaporation solvent method. Erythromycin could be efficiently encapsulated in these microparticles. The releasing of the drug upon incubation in simulated physiological medium occurs by a releasing mechanism largely determined by the hydrodegradation of the host polymer and independent on the amount of loaded drug.

This behavior is looked at being of exceptional interest since liberation rate of the drug could be tuned by adjusting the copolymer composition within the allowed range of solubility in non-water volatile organic solvents.

4.5. References

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