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Polymers and Biopolymers

Modification of Microbial Polyacids for Drug Delivery Systems

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Summary

Polymers are becoming preferred materials in biomedical applications because of their vast diversity of properties, functionalities and applications. Properties as mechanical strength, stability against degradation, biocompatibility and biodegradability, among others, have been attractive for different medical applications. One of the most interesting applications of these materials is drug delivery systems. Biodegradable polymers and copolymers are the preferred materials for the manufacture of a variety of devices for temporal applications in medicine and pharmacology; these biodegradable polymers can be chemically synthesized or biologically produced. Biotechnological polymers have attracted much attention because two main advantages; first, they are produced from renewable resources; and second, as they are biologically produced they are biocompatible, biodegradable and bioresorbable materials. Thus, modification of biotechnological polymers to obtain specific properties or functionalities is a good strategy for the development of promising biomedical materials.

In this Thesis the water soluble biotechnological polymer poly(β,L-malic acid) (PMLA) was modified to change its hydrophilic character to produce non water-soluble polymers capable of forming particulate systems for drug encapsulation and controlled release. PMLA is a polyester-3 with a pendant carboxylic group; it is biocompatible, biodegradable and bioresorbable. The carboxylic side group can be substituted in order to modify the properties of the polymer. The polymer as polyelectrolyte, is also water-soluble.

Different strategies were used for polymer derivatization: direct esterification and amidation through the activation of the carboxylic side groups with carbodiimides; ionic complex formation with a cationic drug (Doxorubicin); and esterification with aliphatic long chains by a two step method employing thiol-ene *click* reactions. Obtained PMLA derivatives resulted in hydrophobic or amphiphilic polymers, which were appropriated for nanoparticle formation, either by emulsion solvent evaporation or by precipitation dialysis techniques.

Derivatives physicochemical characterization was made by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, gel permeation chromatography (GPC) and differential scanning calorimetry. Hydrolytic degradation was followed by GPC and ^{1}H NMR, while particles were observed by scanning electron microscopy and their size and surface charge characterized by dynamic light scattering and ζ-potential measurement. Assays of drug encapsulation and release were also performed and cytotoxicity tests were done on cancer cell lines.

Nanoparticles (100-300 nm aprox.) were obtained from all PMLA derivatives, except for the ionic complex which formed microparticles. Nanoparticles showed potential as drug delivery systems since they were able to encapsulate the anticancer drugs Temozolomide and Doxorubicin, as well as the model drugs Theophylline and Carbamazepine. Drug release was assessed under physiological conditions; the release rate was found to depend on encapsulation method, drug and polymer derivative.

Hydrolytic degradation assays showed that free malic acid and the organic compound derived from the reagent used for modification were the last products of aqueous degradation of PMLA derivatives. Cytotoxicity tests demonstrated the low toxicity of the synthesized derivatives.

Results generated in this Thesis suggest that the biotechnological polymer PMLA is a material of interest as a platform for the design and development of biodegradable drug delivery systems with potential in the therapy of diseases considered today challenging for pharmacologic treatment.

Key words: Poly(malic acid), biotechnological polymer, drug delivery system, biodegradable, biocompatible, nanoparticle, polymer modification, drug encapsulation, cancer, Temozolomide, Doxorubicin.

Resum

Els polímers s'han convertit en els materials preferits per a ús biomèdic degut la gran diversitat de propietats, funcionalitats i aplicacions que tenen. Propietats com resistència mecànica, estabilitat en front de la degradació, biocompatibilitat i biodegradabilitat, entre d'altres, són atractives per a diverses aplicacions mèdiques. Una de les aplicacions més interessants d'aquests materials és com a sistemes d'alliberament controlat de fàrmacs. Els polímers i copolímers biodegradables són els materials predilectes per a la fabricació d'una varietat de dispositius per a aplicacions temporals en medicina i farmacologia; aquests polímers biodegradables poden ser sintetitzats químicament o produïts biològicament. Els polímers biotecnològics han captat l'atenció per dues raons principals; primer, s'obtenen a partir de fonts renovables; i segon, que en ser produïts biològicament són materials biodegradables, biocompatibles i bioassimilables. Per això, la modificació de polímers biotecnològics per obtenir propietats o funcionalitats específiques és una bona estratègia per al desenvolupament d'un material biomèdic prometedor.

En la present Tesi el polímer biotecnològic, soluble en aigua, àcid poli(β,L-màlic) (PMLA) s'ha modificat per canviar el seu caràcter hidrofílic per tal de produir polímers insolubles en aigua capaços de formar sistemes de partícules per a l'encapsulació i l'alliberament controlat de fàrmacs. El PMLA és un polièster-3 amb un grup carboxílic lateral; és biocompatible, biodegradable i bioassimilable. El grup carboxílic lateral pot ser substituït per tal de modificar les propietats del polímer. El polímer com a polielectròlit, és també soluble en aigua.

En aquest treball s'han utilitzat diferents estratègies per a la derivatització del polímer: l'esterificació i l'amidació directes, mitjançant l'activació dels grups carboxílics laterals amb carbodiimides; la formació de complexos iònics amb un fàrmac catiònic (Doxorrubicina); i l'esterificació amb cadenes alifàtiques llargues, utilitzant un mètode en dos passos mitjançant reaccions *clic* tiol-è. Els derivats de PMLA obtinguts van resultar ser polímers hidrofòbics o anfifílics, apropiats per a la formació de nanopartícules, ja sigui pel mètode d'emulsió evaporació de solvent o per la tècnica de precipitació diàlisi.

La caracterització físico-química dels derivats es va realitzar mitjançat espectroscòpia de ressonància magnètica nuclear (RMN) de ^{1}H i ^{13}C, cromatografia de permeació en gel (GPC) i calorimetria diferencial de rastreig. El seguiment de la degradació hidrolítica es va fer mitjançant GPC i ^{1}H RMN, mentre que les partícules van a ser observades amb microscòpia electrònica de rastreig i la seva mida i càrrega superficial caracteritzades mitjançant dispersió de llum i mesurament del potencial-ζ. En van realitzar estudis sobre l'encapsulació de fàrmacs i el seu alliberament, així com assajos de citotoxicitat sobre línies de cèl·lules canceroses.

En van obtenir nanopartícules (100-300 nm aprox.) amb tots els derivats, excepte en el cas dels complexos iònics que formen micropartícules. Les nanopartícules van mostrar potencial com a sistemes d'alliberament controlat ja que va ser possible l'encapsulació dels fàrmacs anticàncer Temozolomida i Doxorrubicina, així com dels fàrmacs models Teofilina i Carbamazepina. L'alliberament de fàrmacs es va avaluar en condicions fisiològiques; la taxa d'alliberament es va trobar dependent del mètode d'encapsulació, del fàrmac i del derivat polimèric utilitzat.

Els assajos de degradació hidrolítica mostren que l'àcid màlic i la molècula orgànica derivada del compost utilitzat per a la modificació són els últims productes de la degradació hidrolítica dels derivats de PMLA. Les proves de citotoxicitat demostren la baixa toxicitat dels derivats sintetitzats.

Els resultats generats en aquesta Tesi suggereixen que el polímer biotecnològic PMLA és un material d'interès com a plataforma per al disseny i desenvolupament de sistemes biodegradables d'alliberament controlat de fàrmacs amb potencial en la teràpia de malalties considerades avui dia un repte per el tractament farmacològic.

Paraules clau: Àcid polimàlic, polímer biotecnològic, sistema d'alliberament controlat, biodegradable, biocompatible, nanopartícula, modificació de polímers, encapsulació de fàrmacs, càncer, Temozolomide, Doxorrubicina.

Resumen

Los polímeros se han convertido en los materiales preferidos para usos biomédicos debido a la gran diversidad de propiedades, funcionalidades y aplicaciones que poseen. Propiedades como resistencia mecánica, estabilidad a la degradación, biocompatibilidad y biodegradabilidad, entre otras, son atractivas para diversas aplicaciones médicas. Una de las aplicaciones más interesantes de estos materiales son los sistemas de liberación controlada de fármacos. Los polímeros y copolímeros biodegradables son los materiales predilectos para la fabricación de una variedad de dispositivos de uso temporal en medicina y farmacología; estos polímeros biodegradables pueden ser sintetizados químicamente o producidos biológicamente. Los polímeros biotecnológicos han captado gran atención por dos razones principales; primero, son obtenidos a partir de recursos renovables; y segundo, que al ser biológicamente producidos, estos son biodegradables, biocompatibles y bioasimilables. Por esto, la modificación de polímeros biotecnológicos para la obtención de propiedades o funcionalidades específicas es una buena estrategia para el desarrollo de un material biomédico prometedor.

En la presente Tesis el polímero biotecnológico, soluble en agua, acido poli(β,L-málico) (PMLA) se modificó para cambiar su carácter hidrofílico para producir polímeros insolubles en agua capaces de formar sistemas particulados para la encapsulación y liberación controlada de fármacos. El PMLA es un poliéster-3 con un grupo carboxílico lateral; es biocompatible, biodegradable y bioasimilable. El grupo carboxílico lateral puede ser substituido para modificar las propiedades del polímero. El polímero como polielectrolito, es también soluble en agua.

En este trabajo se utilizaron diferentes estrategias para la derivatización del polímero: la esterificación y amidación directas, mediante la activación de los grupos carboxílicos laterales con carbodiimidas; la formación de complejos iónicos con un fármaco catiónico (Doxorrubicina); y la esterificación con cadenas alifáticas largas, utilizando un método de dos pasos mediante reacciones *click* tiol-eno. Los derivados de PMLA obtenidos resultaron ser polímeros hidrofóbicos o anfifílicos, apropiados para la

formación de nanopartículas, ya sea por el método de emulsión evaporación de solvente o por la técnica de precipitación diálisis.

La caracterización fisicoquímica de los derivados se realizó mediante espectroscopia de resonancia magnética nuclear (RMN) de ^{1}H y ^{13}C, cromatografía de permeación en gel (GPC) y por calorimetría diferencial de barrido. La degradación hidrolítica fue monitoreada mediante GPC y ^{1}H RMN, mientras que las partículas fueron observadas con microscopía electrónica de barrido y su tamaño y carga superficial caracterizadas mediante dispersión de luz y medición del potencial-ζ. Estudios sobre la encapsulación de fármacos y su liberación fueron realizados, así como ensayos de citotoxicidad sobre líneas de células cancerígenas.

Se obtuvieron nanopartículas (100-300 nm aprox.) con todos los derivados, excepto para el complejo iónico que formaron micropartículas. Las nanopartículas mostraron potencial como sistemas de liberación controlada ya que fue posible la encapsulación de los fármacos anticanerígenos Temozolomida y Doxorrubicina, así como de los fármacos modelos Teofilina y Carbamazepina. La liberación de fármacos se evaluó en condiciones fisiológicas; la tasa de liberación se encontró dependiente del método de encapsulación, el fármaco y el derivado polimérico utilizado.

Los ensayos de degradación hidrolítica muestran que el ácido málico y la molécula orgánica derivada del compuesto utilizado para la modificación son los últimos productos de la degradación hidrolítica de los derivados de PMLA. Las pruebas de citotoxicidad demostraron la baja toxicidad de los derivados sintetizados.

Los resultados generados en esta Tesis sugieren que el polímero biotecnológico PMLA es un material de interés como plataforma para el diseño y desarrollo de sistemas biodegradables de liberación controlada de fármacos con potencial en la terapia de enfermedades consideradas hoy día un reto para el tratamiento farmacológico.

Palabras clave: Acido polimálico, polímero biotecnológico, sistema de liberación controlada, biodegradable, biocompatible, nanopartículas, modificación de polímeros, encapsulación de fármacos, cáncer, Temozolomide, Doxorrubicina.

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Acronyms

°C	Celsius degrees
AA	Amino acid
Abs	Absorbance
AIC	5-aminoimidazole-4-carboxamide
AGAUR	Agency for administration of university and research grants (by its Spanish initials)
ATCC	American type culture collection
ATR	Attenuated total reflectance
BBB	Blood brain barrier
CBZ	Carbamazepine
cm	Centimetre
CONACyT	National science and technology council (by its Spanish initials)
***co*PGGA-Al$_x$H$_y$**	***co***Poly(α-allyl-β,L-glutamate-*co*-glutamic acid)
***co*PGGA-PrSOc$_x$H$_y$**	***co***Poly(α-3-octylthio-propyl-β,L-glutamate-*co*-glutamic acid)
***co*PGGA-PrSdoD$_x$H$_y$**	***co***Poly(α-3-dodecylthio-propyl-β,L-glutamate-*co*-glutamic acid)
***co*PGGA-PrShxD$_x$H$_y$**	***co***Poly(α-3-hexadecylthio-propyl-β,L-glutamate-*co*-glutamic acid)
***co*PMLA-Al$_x$H$_y$**	***co***Poly(α-allyl-β,L-malate-*co*-malic acid)
***co*PMLA-Bu$_x$H$_y$**	***co***Poly(α-butyl-β,L-malate-*co*-malic acid)
***co*PMLA-Et$_x$H$_y$**	***co***Poly(α-ethyl-β,L-malate-*co*-malic acid)
***co*PMLA-PrSOc$_x$H$_y$**	***co***Poly(α-3-octylthio-propyl-β,L-malate-*co*-malic acid)
***co*PMLA-PrSdoD$_x$H$_y$**	***co***Poly(α-3-dodecylthio-propyl-β,L-malate-*co*-malic acid)
***co*PMLA-PrShxD$_x$H$_y$**	***co***Poly(α-3-hexadecylthio-propyl-β,L-malate-*co*-malic acid)
Da	Dalton

DAPI	4',6-diamidino-2-phenylindole
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexylurea
DDS	Drug delivery system
DLS	Dynamic light scattering
DMPA	2,2-Dimethoxy-2-phenylacetophenone
DMSO	Dimethylsulfoxyde
D_2O	Deuterated water
DOX	Doxorubicin
DSC	Differential scanning calorimetry
EE	Encapsulation efficiency
Et	Ethyl
EPR	Enhanced permeation retention
F	Phenylalanine
FDA	Food and drug administration
FT-IR	Fourier transform infrared spectroscopy
GPC	Gel permeation chromatography
GRAS	Generally regarded as safe
h	Hour
HPLC	High performance liquid chromatography
HFIP	Hexafluoro-2-propanol
I	Ionic strength
L	Leucine
Leu	Leucine
MHz	Megahertz
k	Kilo (10^3)
µg	Microgram
µm	Micrometre
µM	Micromolar
M	Molar
MATGAS	Materials and Gases
Me	Methyl

MEM	Minimum essential medium
mg	Milligram
MICINN	Science and innovation ministry (by its Spanish initials)
min	Minute
mL	Milliliter
MLABn	Benzyl malolactonate
mm	Millimetre
mM	Millimolar
mmol	Millimol
ms	Millisecond
mV	Millivolt
M_w	Molecular weight (weight average)
MWCO	Molecular weight cut-off
MTS	Yellow [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]
NEAA	Non essential amino acids
NIH	National institutes of health
nm	Nanometre
NMP	N-methyl-pyrrolidone
NP	Nanoparticle
NMR	Nuclear magnetic resonance
O/W	Oil/water
PAALM-1	Poly(α-methyl-β,L-malate)
PAALM-L-X	***co***Poly(α-leucine ethyl ester-β,L-malate-*co*-malic acid)
PAALM-F-X	***co***Poly(α-phenylalanine methyl ester-β,L-malate-*co*-malic acid)
PB	Phosphate buffer
PC	Poly(ε-caprolactone)
Pd.	Polydispersity
PEG	Polyethylene glycol

PGA	Poly(glycolic) acid
PGGA	Poly(γ-glutamic) acid
pH	Potential of hydrogen
PHA	Poly(hydroxyalkanoate)
Phe	Phenylalanine
pK_a	Acid dissociation constant
PLA	Poly(lactic acid)
PLG	Poly(lactide-*co*-glycolide)
PLGA	Poly(lactic-*co*-glycolic acid)
PMLA	Poly(β,L-malic acid)
PMLA-Bn	Poly(α-benzyl-β,L-malate)
PMLA-Bu	Poly(α-butyl-β,L-malate)
PMLA-Et	Poly(α-ethyl-β,L-malate)
PMLA-NHS	Poly(α-N-hydroxysuccinimidyl-β,L-malate)
ppm	Parts per million
PVA	Poly(vinyl alcohol)
ROP	Ring opening polymerization
RT	Room temperature
s	Second
SEM	Scanning electron microscopy
TEA	Triethylamine
TEO	Theophylline
T_g	Glass transition temperature
TCA	Tricarboxylic acid
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TMZ	Temozolomide
UV-Vis	Ultraviolete-visible
v	Volume
w	Weight
W	Watt

1

Introduction

Knowledge concerning how to obtain, process and use materials over time has been a key element for the civilization progress. At the beginning, there was the rock era in where the first tools were developed; later the metal era which allowed more sophisticated instruments and afterward the industrial revolution. Now we are witnessing the era of synthetic materials that is predominated by polymeric materials which are going beyond traditional materials with the possibility of creating *smart* materials.

Polymers are becoming preferred materials because of their vast diversity of properties, functionalities and applications. A relevant area that has put a lot of interest in polymeric materials is the medic field; properties as mechanical strength, stability against degradation, biocompatibility and biodegradability, among others, have been attractive for different medical applications. One of the more interesting applications is drug delivery systems, which in the last decades have presented a fast expansion because of the progress in our knowledge in different areas like molecular biology, biotechnology, nanotechnology, pharmacology and material science. The design and development of new biodegradable materials with specific functionalities is in the scope of researchers to achieve an effective and efficient system for controlled drug release.

Among these new polymeric materials we can make a two major class subdivision, those chemically synthesized and those from

biological origin which are produced biotechnologically. The last ones have two main advantages; first they are produced from renewable resources; and second, because they are biologically produced they are biocompatible, biodegradable and bioresorbable materials. Thus, modification of biotechnological polymers to obtain specific properties or functionalities is a good strategy for the development of a promising biomedical material.

One of these promising biotechnological polymers is poly(β,L-malic acid) (PMLA), which has been recently under research for biomedical applications as it has been classified as biocompatible, non-immunogenic and bioresorbable material. For drug delivery systems the most prominent system has been developed as a soluble conjugate, Polycefin, which consist in a multifunctional macromolecule with PMLA as a backbone and different pendant functional moieties (*i.e.* PEG chains, monoclonal antibodies, fluorescents probes, bioactive compounds, membrane disrupting groups) for the treatment of brain and breast cancer. In our group we have been working in the development of solid particulate delivery systems; first works were made with methylated PMLA which evidenced the potential of PMLA for particle formation; nevertheless, methanol released from this derivative could result in cell toxicity. The main focus of this Thesis is the generation of low cytotoxic derivatives, from the modification of biotechnologically produced PMLA, and the exploration of a post-polymerization modification technique which could allow multi-functional derivatization of PMLA for solid nanoparticulated systems for controlled drug delivery.

1.1. General objective

Modification of the biotechnological polymer poly(β,L-malic acid), by the shifting of its hydrophilic/hydrophobic character, for preparation of nanoparticles capable of drug encapsulation with potential as drug delivery systems.

1.2. Specific objectives

i. Formation and characterization of poly(α-methyl β,L-malate) nanoparticles for Temozolomide and Doxorubicin encapsulation.

ii. Synthesis and characterization of poly(α-ethyl β,L-malate) and poly(α-butyl β,L-malate) 50 and 100 % modified, nanoparticle formation by two different methods for Temozolomide and Doxorubicin encapsulation.

iii. Grafting of leucine ethyl ester and phenylalanine methyl ester on poly(β,L-malic acid), characterization and nanoparticle formation for Temozolomide and Doxorubicin encapsulation.

iv. Ionic complex formation and characterization between poly(β,L-malic acid) and Doxorubicin, for a pH dependent drug release system.

v. Synthesis and characterization of comb-like polymers by grafting long aliphatic chains through thiol-ene c*lick* reactions on poly(β,L-malic acid), nanoparticle formation and drug encapsulation. A comparative study with poly(γ-glutamic acid).

1.3. Outline

In this Thesis the water soluble biotechnological polymer, poly(β,L-malic acid), was modified to shift its hydrophilic character to produce a non water-soluble polymer capable of forming particulate systems for drug encapsulation and controlled release. The present work is divided in nine chapters as follows:

Chapter 1- The current chapter presents a general introduction, aims and organization of the Thesis.

Chapter 2- This chapter attends to a review of the polymers and techniques used in this Thesis with special focus on PMLA and its derivatization for biomedical applications.

Chapter 3- In this section the general materials and methods used for the research are summarized.

Results of the Thesis are divided in chapters which correspond to different modification strategies, as follows:

Chapter 4- This chapter presents a study of nanoparticle formation by emulsion solvent-evaporation method of poly(α-methyl β-malate) with different surfactants. PMLA was previously methylated with diazomethane, hydrolytic degradation and cytotoxicity were studied and Temozolomide and Doxorubicin encapsulated.

Chapter 5- This part treats about the esterification of PMLA with ethanol and 1-butanol to obtain hydrophobic homopolymers and amphiphilic copolymers. Polymers characterizations, hydrolytic degradation, nanoparticles formation by two different methods, drug encapsulation and release are presented, as well as cytotoxicities and cellular uptake assays are discussed.

Chapter 6- This chapter deals with the modification of PMLA with hydrophobic amino acids with the aim of making amphiphilic molecules capable of self-assembling into nanoparticles for drug encapsulation. Results include polymer characterization, hydrolytic degradation, particle formation, drug encapsulation and cytotoxicity studies.

Chapter 7- This section makes reference to the ionic complexation of PMLA with the cationic drug Doxorubicin. The change of hydrophilicity for particle formation is directly driven by the drug complexation on the polymer. Characterization of the complex, hydrolytic degradation and drug release are presented.

Chapter 8- This chapter presents the synthesis of comb-like copolymers of PMLA and PGGA as a comparative study, through the grafting of aliphatic long lateral chains by thiol-ene *click* reactions. Amphiphilic copolymers are able to self-assemble in particles for encapsulation of drug models. Characterization, hydrolytic degradation, particle formation and drug encapsulation/release studies are presented.

Chapter 9- Last chapter summarize the general conclusions of the performed research.

2

Biodegradable Polymers for Drug Delivery Systems

2.1. Introduction

The history of materials in medicine goes far into the past time to the Egyptian culture, which used linen sutures to close large wounds. Nowadays, material and medical sciences have evolved into a new age of medical materials or biomaterials. There are two main kinds of materials that are going to be introduced in a body, those for prosthetic purposes and those whose contribution is required for a limited period of time (Figure 2.1). For many years, stable materials were used without distinction between permanent and time-limited applications. Polymers became interesting materials for biomedical applications because their mechanical properties and diverse functionalities. Long time ago, compounds derived from biopolymers like animal sinews were used, but the discovery of fiber forming and hydrolysability of poly(glycolic acid) (PGA) was the first step towards the development of a synthetic biodegradable and bioresorbable polymer for time-limited applications.[1]

The history of synthetic degradable and biodegradable polymers started in 1960s when Schmitt and Polestina, at Davis & Geck, synthesized and patented poly(glycolic acid) for use it as biodegradable suture. Ethicon added lactic acid to the composition to make the biodegradable poly(lactic-*co*-glycolic acid) (PLGA).[2] It was realized that the water sensitive aliphatic polyesters derived from glycolyde and

lactide could be of interest to process therapeutic aids having lifetimes comparable with the healing time of injured soft and hard tissues with great potential for a variety of medical applications.[3] Around the 70s years the polymers evaluated as biomaterials had increased rapidly (Table 2.1), however the number of compounds that have reached commercial application is still small.[4]

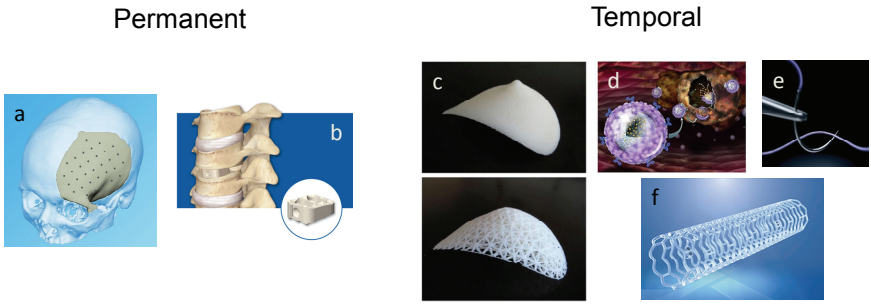


Figure 2.1. Permanent and temporal medical applications of polymers: a) bond prosthesis, b) joint prosthesis, c) tissue regeneration scaffolds, d) drug delivery systems, e) chirurgical sutures and f) arterial stents.

Within the last decades a variety of natural and synthetic materials has been extensively studied for possible biomedical applications, such as bone or joint prosthesis, artificial blood vessels and surgical sutures, among others. However, one of the most attractive potential uses of these polymers is in the formulation of novel drug delivery systems for parenteral administration.[5] Here, the polymeric material is an essential part of a therapeutic system which is designed to achieve either control over drug delivery rate, temporal control, or for a selective delivery at a specific site of action, spatial control.[6]

Table 2.1. Polymers in biomedicine and their applications.

Polymer family	Origen	Degradation products	Applications
Polyesters: (Poly: lactic acid, glycolic acid, malic acid, hydroxy acids, caprolactone)	Chemical/natual	Lactic acid Glycolic acid Malic acid Hydroxy acids	Sutures, Stents Drug delivery Tissue scaffolds
Polysaccharides: (Chitosan, Alginates)	Chemistry on chitin	Unknow	Drug delivery Hydrogels
Polyamides: (Poly: glutamic acid, lysine, aspartic acid)	Chemical/natural	Glutamic acid	Drug delivery Hydrogels Sutures
Polyanhydrides	Chemical	Chemicals	Surgery and pharmacology
Poly(orthoesters)	Chemical	Chemicals	Drug delivery
Poly(methyl methacrylate)	Chemical	Non-biodegradable	Drug delivery Prosthesis Contact lenses Fracture fixation
Polytetrafluoroethylene	Chemical	Non-biodegradable	Heart valves Vascular grafts Nerve repair

Conventional pharmacotherapy involves the use of drugs whose absorption and therefore bioavailability depends on many factors, such as solubility, pK_a, molecular weight and chemical stability. In general, especially their low molecular weights, confers them the capacity to cross various body compartments and access numerous cell types and subcellular organelles. However, this form of indiscriminate distribution leads to the occurrence of side effects and the need of higher doses of the drug to achieve a satisfactory pharmacological response. Thus, the success of medical treatments not only depends on the therapeutic agent, but also in its bioavailability in the site of action inside the body.[7] DDS seeks to improve the pharmacological activity of drugs by enhancing absorption, distribution, metabolism and excretion (bioavailability); they

are usually high molecular weight carriers such as nano and micro particles, capsules, capsosomes, micelles and dendrimers, in which the drug is embedded or covalently bound.[8] These systems have been used for the transport and delivery of macromolecules like peptides and proteins,[9,10] gens,[11,12] vaccines[13,14] and drugs of low molecular weight.[6]

Drug delivery systems start to develop in the late 60s, Dupont researchers' added peptide drugs to PLA and fabricated microparticles and pellets for DDS; other laboratories were making clinical tests on PLGA microencapsulated steroids for a contraceptive method. First commercialized DDS were macroscopic devices, like the ophthalmic insert called the Ocusert® that released the anti-glaucoma drug pilocarpine or the different contraceptive subcutaneous implants.[2] The pioneer work of Langer and Folkman, who showed that proteins could be released from non-degradable polymers matrices[15] may have stimulated to think about other ways to delivery such drugs, such as by loading them in biodegradable polymers. Other biodegradable polymers started to be developed like poly(ethylene glycol terephtalate), polyorthoesters, polyanhydrides and block copolymers of PLGA-PEG.

In the late 70s the concept of polymer-drug conjugate or nano-therapeutics became on the table. Three concepts were key factors for the development of nano-therapeutics. First, the concept of PEGylation, which refers to poly(ethylene glycol) conjugated with drugs or vehicles to enhance blood circulation times; second, the concept of active targeting by the use of cell recognition molecules in the DDS; and third, the enhanced permeation retention (EPR), where nano-scale carriers are entrapped in solid tumors due to the leaky vasculature of the fast growing tumor, which is also called passive targeting.[2,16] Since then a lot of formulations have been designed and tested, micelles, liposomes, nanoparticles, conjugates, for the transport and delivery of hydrophilic and hydrophobic drugs. It is possible to classify two types of polymeric carriers, macromolecular-conjugates carriers, where the drug is covalently linked to a macromolecule soluble in body fluid; and colloidal

carriers, where the active compound is entrapped in a solid matrix formed from a macromolecule.[5] The first clinically approved injectable degradable microparticulate DDS was Decapeptyl® LP, a treatment for prostate cancer, launched in 1986 in Europe and still in the market.[2] At nanoscale, only some drug-loaded liposomes like Doxil® had reached its application. Figure 2.2 shows the time line of DDS development.

Nanotechnology and drug controlled release have become important since the USA initiative in the year 2000, in which they dedicated more than 20 research centers to these topics. Five of them were designated to the development of what some ones consider the future of the pharmaceutical industry, and that the prognostics see the technology for drug controlled release at nano level as reality in 2020. But the development of nanotechnology has brought considerable attention to the problem of toxicity of nanomaterials, because of its unique physicochemical properties. A crucial feature of these systems is the mechanism by which they are removed from the body; they may be excreted directly via renal clearance or biodegraded and metabolized. The problem has been addressed in many cases by the development of biodegradable polymers, optimization of chemical composition, surface modification and other approaches.[17]

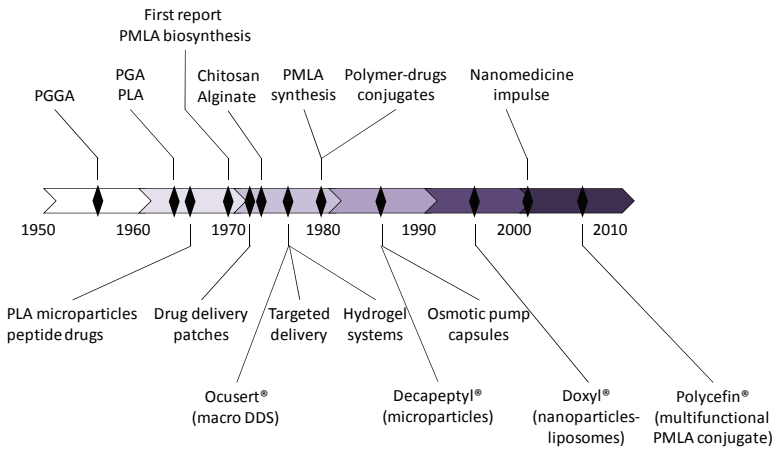


Figure 2.2. Time line of DDS development.

Nowadays, biodegradation and absorption of any high molecular weight material which is to be introduced in the body for a limited period of time must be considered as a prerequisite for applications in human therapy;[18] considering this, biodegradable polymers and copolymers are the preferred materials for the manufacture of a variety of devices that are today widely applied in medicine and pharmacology;[19] specifically they have become increasingly important in the development of DDS, for which they must meet very specific requirements: *a*) biocompatibility of the polymer and its degradation products, *b*) sufficient mechanical strength to meet the needs of specific applications, *c*) degradation kinetics matching the biological process, *d*) solubility in various solvents, *e*) chemical, structural and application versatility, *f*) economically acceptable shelf life and *g*) approval of the European Medicine Evaluation Agency or from the Food and Drug Administration (USA).[20]

We must differentiate among the terms bioabsorbable, biodegradable and bioresorbable (bioassimilable); bioabsorbable refers to a polymeric material or device that can dissolve in body fluids without any molecular mass decrease. Biodegradable is used for polymer materials which break down to macromolecule degradation but no proof of elimination from the body. And last bioresorbable, in which is assumed the complete elimination of the initial foreign material and of degradation products with no residual effects.[4]

Latest advances in DDS are based on the use of biopolymers or biodegradable synthetic polymers, which allow repeated human treatment without deposition and storage diseases. This has stimulated the modification of naturally occurring biopolymers and the development of new synthetic ones.[20,21] In general, research and production of biopolymers and metabolite-based polymers have captured great interest by the elucidation of its potential in the biomedical field, because of their biological origin they are *a priori* considered as a biodegradable and bioresorbable by the human body.[22]

The most studied and used polymeric materials in biomedicine are the polyesters poly(lactic acid), poly(glycolic acid), polycaprolactone (PC) and their copolymers.[7] Other new biodegradable polymers have been studied like polyamides, polyanhydrides, polyorthoesters and biotechnological polymers; among these there are two biopolymers that present great advantage over PLA and PGA, poly(β-malic acid) (PMLA) and poly(γ-glutamic acid) (PGGA). It is the functionality that the lateral carboxylic group confers to them, which allows their relatively easy and direct chemical modification, to modulate properties like their solubility, degradation rate, targeting molecules insertion, stability and release of encapsulated drugs, among others.

Even though significant advances have been made in the field of micro and nano DDS, there are still many challenges in this field, like standardized evaluation methods and newer site directed polymers.[23] There is an interconnection between the development of DDS in the future and the increment of our knowledge in biology; the better we know how the live systems work, the easier it will be to design nano-scale DDS that are serum stable, efficiently taken by specific cells, able to escape the endosomes and target specific sites or pathways inside the cell. Polymer based DDS are an attractive area with innumerable opportunities for further research and development; however, the success in these areas depends on the intense efforts of scientists from different disciplines such as biology, pharmacy and polymer science (Figure 2.3).[7]

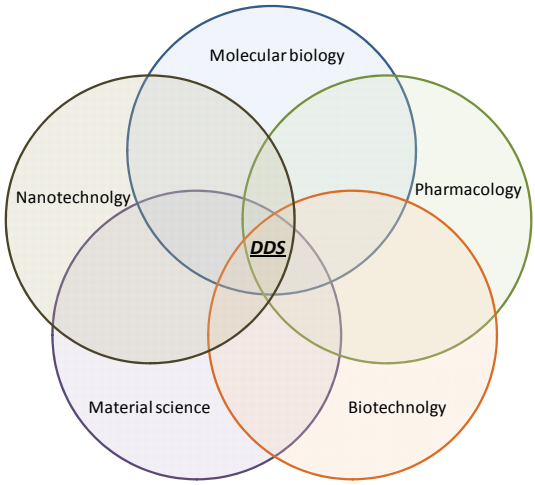


Figure 2.3. Disciplines involved in drug delivery systems development.

2.2. Biotechnological polymers: poly(β,L-malic acid)

2.2.1. Chemical structure and physicochemical properties

Poly(β,L-malic acid) belongs to the polyester family, it is derived from malic acid (α-hydroxy succinic acid), a chiral hydroxy acid whose L enantiomer is a natural bioorganic compound present in a great variety of fruits and vegetables and a mammal metabolite of the tricarboxylic acid cycle (TCA). It is linked by ester bonds which are formed between the hydroxyl group and the carboxyl group located at the β position (polyester 3); in difference with most of the polyesters it has a lateral carboxylic group pendant from the repetitive unit α-carbon, giving an asymmetric character to this carbon which is naturally found as stereo- and regio- regular polymer only in L configuration; thus it is an optically active polymer. PMLA can be produced by microorganism fermentation[24] and also chemically synthesized by direct polycondensation or by ring opening polymerization making possible to obtain poly(α,L-malic acid) or poly(β,L-malic acid) or a copolymer of both (Figure 2.4).[20]

PMLA is highly hygroscopic and water soluble, in both its acid and salt forms. Particularly, PMLA from *Physarum polycephalum* is obtained as a white semi-crystalline powder, soluble in most polar solvents. It is a semi-crystalline polymer with a melt temperature around 210 °C and a thermal decomposition close to the melting of the polymer which evolves by an unzipping depolymeraziton mechanism with generation of fumaric acid.[25] It is a weak acid with a pK_a between 3 and 4 depending in its origin. All carboxylic groups are ionized at neutral pH, thus the polymer is highly charged under physiological conditions.[26] The lateral carboxylic group of PMLA gives it a polyelectrolyte character, which ionizes readily in water and makes it different from other natural polyesters like poly(hydroxyalkanoates);[4] due to the reactivity of these groups the chemical modification or derivatization to obtain different materials with specific physicochemical properties is possible. The charge repulsion of the ionized polymer provokes that an extended conformation is preferred by PMLA, moreover, the polyanion has a high degree of conformational freedom in aqueous solution so it does not display higher-ordered structures in water.[24]

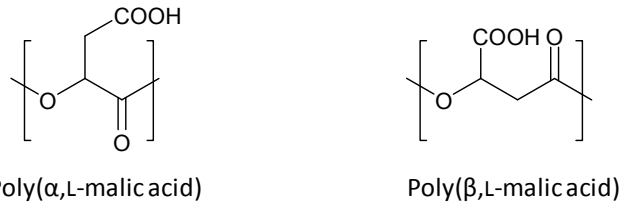


Figure 2.4. Chemical structure of poly (malic acid) stereoisomers.

2.2.2. Biological synthesis

The first published work on natural PMLA is about low molecular PMLA production from *Penicillium cyclopium* in 1969,[27] however the chemical synthesis from the corresponding lactones[28] was done before the biological synthesis was recognized.[29] Since then low and high molecular weight PMLA have been obtained from various myxomycetes and several mitosporic fungi like *Physarum polycephalum* (Table 2.2). PMLA biosynthesis has been related to DNA replication regulatory system, because it forms specific complexes with DNA polymerases to inhibit their activity.[30] Biological systems only synthesizes enantiomeric pure PMLA, with β structure and L configuration,[31] result of the esterification between the hydroxyl group and the β-carboxyl from the malic acid monomeric unit. PMLA architecture can be linear or branched depending upon the microorganism that produce it.[24] Polymer production depends on several factors such as the composition of the growth medium, the time of harvest and the particular stage of the life-cycle of the organism under consideration. PMLA from *Physarum polycephalum* varies widely in molecular mass (10-300 kDa) while that from mitosporic fungi is considerably smaller (5-9 kDa).[32]

Table 2.2. PMLA producing microorganisms.

Organism	Polymer content[a] (mg·L^{-1})	M_w (kDa)	D^b
Aureobasidium sp.[33,34]	7.8 – 62.2 x 10^3	5 – 11	1.1
Physarum polycephalum[33]	400 – 2.7 x 10^3	10 – 300	1.2 – 3.7
Penicillium cyclopium[24]	5.7 x 10^3	5	
Cladosporium sp.[24]	24 – 350		

[a] PMLA concentration in the culture medium.
[b] Molecular weight dispersity of the polymer (M_w/M_n).

Of the various forms of cells in the fungi life cycle only the plasmodium produce PMLA, neither the amoebae nor spherules nor spores do contain PMLA (Figure 2.5). Polymer is first produced in the cell nuclei for a later release into the growth medium via the cytosol. PMLA is produced from D-glucose involving the citric acid cycle for the production of precursors; the amount of polymer released to the medium is proportional to the content of D-glucose in the culture medium.[24] It has been shown that $CaCO_3$ strongly stimulate PMLA production, and that this production it is not cell-growth dependant but competitive for glucose as carbon source.[32]

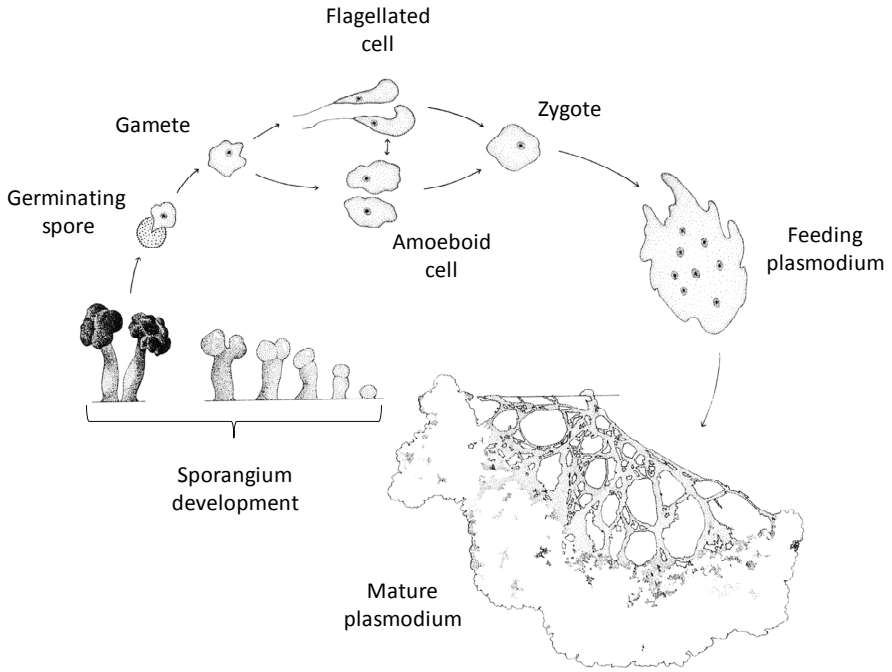


Figure 2.5. Life cycle of the myxomycete *Physarum polycephalum*. Drawing by Margret LaFarge.[35].

2.2.3. Chemical synthesis

On another hand, chemical synthesis of PMLA is also possible. In fact, it has been the synthetic PMLA which has been used in most of the research about this polymer, because it is possible to control its molecular weight. PMLA has been synthesized in several steps from bromosuccinic acid,[36] aspartic acid[37] and malic acid enantiomers.[38] Chemical synthesis can be done by two different routes, polycondensation of L-malic acid (Figure 2.6a) which leads to a low molecular weight polymer[39] and ring opening polymerization (ROP) of substituted β-lactonates[20,28,31] (Figure 2.6b) which allows configuration control and high molecular weight polymers. The first high molecular weight PMLA synthesis was made by ROP by Vert and Lenz in 1979 after the successful synthesis of the β-substituted β-malolactones, such as benzyl β-malolactonate.[28] The biochemical reactivity of synthetic PMLA has been indistinguishable from natural polymer.[40]

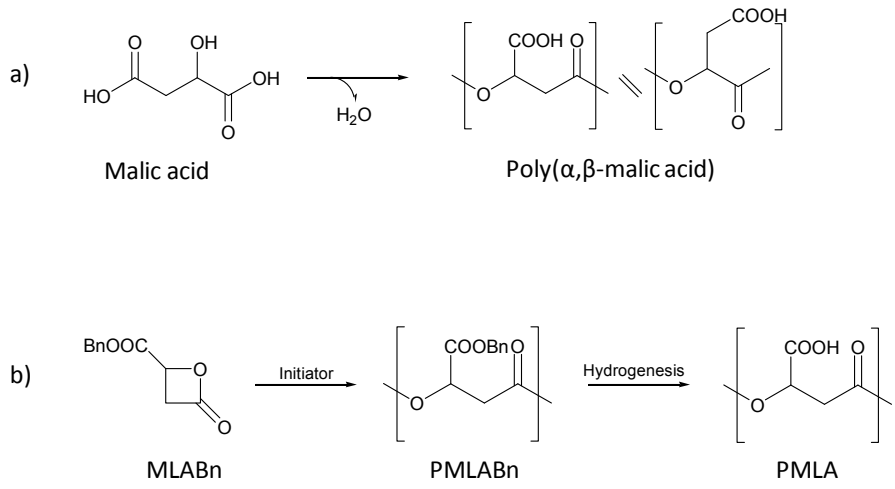


Figure 2.6. Chemical polymerization of poly(malic acid): a) L-Malic acid polycondensation and b) ring opening polymerization of β-malolactonate (MLABn) for racemic PMLA synthesis.

2.2.4. Degradability

Because of the PMLA potential as material for biomedical applications PMLA and its derivatives hydrolytic degradation have been extensively studied. PMLA hydrolytic degradation happens spontaneously or by enzymatic hydrolysis,[24] and depending on the pH degradation can be substantial due to the autocatalytic degradation, which results in a non-random cleavage of the main chain.[5,26] The rate of hydrolysis has its minimum in phosphate buffered neutral solutions, which should be used for the purification and storage of polymalate salts. Hydrolytic degradation of the polymer sodium salt at pH 7.0 and 37 °C results in a random cleavage of the polymer, the molecular mass decrease by 50 % after a period of 10 h.[41] The end product of PMLA hydrolytic degradation is L-malic acid one of the metabolites of the tricarboxylic acid cycle (Figure 2.7), thus it is considered a bioresorbable (bioassimilable) polymer.[4] In respect to its derivatives, alkyl esters, previous studies have shown that degradation rate is in function of polymer composition, as more hydrophobic groups are substituted on the polymer the lower the degradation rate is.[42] In a different work Martínez-Barbosa *et al.* studied the hydrolytic degradation on synthetic PMLA derivatives, concluding that degradation rate is directly related to material hydrophilicity.[21] Nevertheless, degradation mechanisms in biological systems are very different, since there are a lot of enzymes involved,[43] where degradation rate will significantly depend on the concentration and activity of those biocatalyzers. PMLA biodegradation and bioassimilation has been also studied *in vivo* through radio-labeling, founding that its blood clearness is fast and that the major part is excreted via urinary in small oligomers form.[44] Moreover, citotoxicity tests have been done to a wide variety of derivatives founding that most of them are well tolerated by the tested cell lines.[21,45-47] Due to these characteristics and because PMLA hydrolytic degradation product is malic acid, PMLA is considered as biodegradable, non-immunogenic, non-toxic and bioresorbable material for mammals and humans.[4,26]

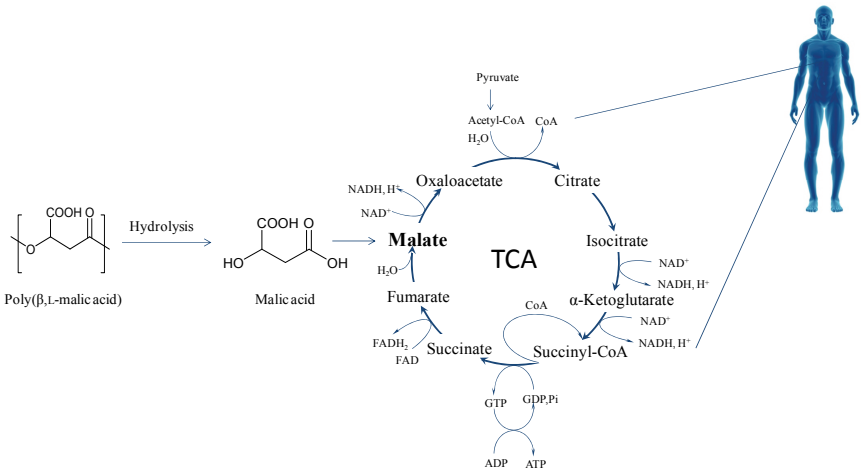


Figure 2.7. Malic acid: PMLA hydrolytic degradation product and a metabolite of mammals' tricarboxylic acid cycle.

2.2.5. PMLA modification

PMLA main chain bears lateral carboxylic groups and a terminal hydroxyl and carboxyl groups which can be substituted, allowing the modulation of the overall polymer hydrophobicity[21,48] or the introduction of bioactive ligands to give specific properties to the polymer.[49,50] PMLA has three groups for modification: A) carboxyl groups can be reacted in the presence of carbodiimides as coupling reagents, B) the terminal hydroxyl group via the reaction of isothiocyanates, and C) the β-carboxyl of the main chain by the attack of a nucleophile, such as primary amines and diamines, leading to simultaneous chain scission (Figure 2.8).[26] The high hygroscopicity and susceptibility to hydrolysis of PMLA has motivated the search of new derivatives with better stability and lower degradation rates, as well as derivatives that can be useful for nanoparticle formation and drug encapsulation. Esterification of the carboxyl side group of PMLA results in polymers with a dramatic change in its original properties. Our group had previously reported the methylation of biological PMLA by reaction with diazomethane,[51] resulting in a highly crystalline and highly

hydrophobic polymer in difference with the hydrophilic PMLA; and the formulation of microparticles for erythromycin delivery in which methylated PMLA particles are considered promising devices for controlled delivery of several drugs.[48,52]

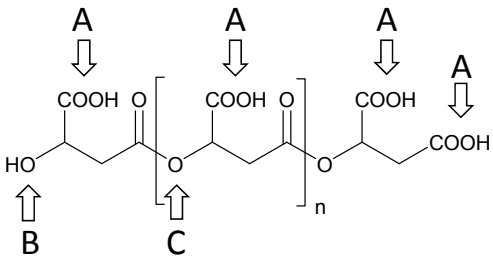


Figure 2.8. PMLA groups susceptible to modification. A) carboxyl groups with carbodiimides as coupling reagents; B) terminal hydroxyl group via isothiocyanates; C) ester group by the attack of a nucleophile (to yield two shortened polymer molecules and a substitution at the terminal carboxyl group of one of them).

Other derivatives have been studied, but these are not prepared by postpolymerization modification techniques. Instead, they are obtained directly by ROP polymerization of different modified β-malolactones and the partial or total hydrogenolysis of copolymers with poly(β-benzyl malate). With this technique derivatives like poly(β-benzyl malate), poly(β-butyl malate), poly(β-hexyl malate), and copolymers have been obtained.[21,53] Furthermore, the copolymerization of malic units with lactic units has been performed by ROP in the presence of stannous octoate, these has allowed the functionalization of PLA while its hydrophilicity increases.[54]

2.2.6. Biomedical applications

PMLA and its derivatives has been used as platform in the synthesis of nanocarriers for drug delivery systems in which the active compound has been covalently attached[18,55] or physically entrapped;[21,48,53,56] water insoluble PMLA-Bn and other hydrophobic derivatives has been used to prepare various solid devices, including compression molded pellets, films, microparticles and nanoparticles but none of these have yet led to concrete therapeutic application.[20,57] Also PMLA has been investigated as a constituent for macromolecular conjugates bearing several functionalities to treat human brain and breast tumors in mouse models.[50,58,59] In all of these investigations it has been concluded that PMLA is a promising building block for the design of efficient drug delivery systems.

Most prominent PMLA formulations are clofazimine encapsulation for intravenous treatment of tuberculosis,[60] and Polycefin, from Cedars-Sinai Medical Center research group, a biopolymeric nanoconjugate for breast and brain cancer treatment with effective targeting delivery by conjugation of tumor targeting antibodies;[61] which has been also conjugated with Temozolomide,[62] Doxorubicin[55] and antisense oligonucleotides[50,59] as active anticancer compounds. It has been shown *in vivo* that the nanoconjugate can effectively reach the tumor tissue and reduce tumor growth in rats (Figure 2.9).

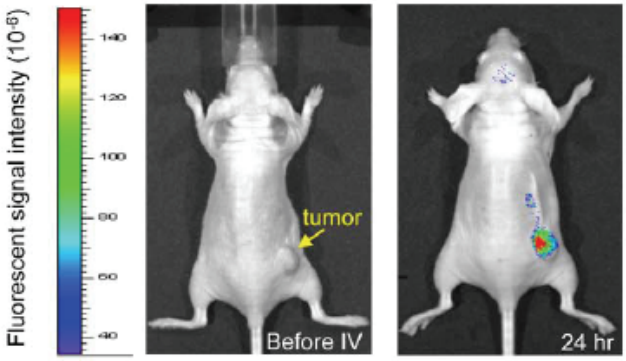


Figure 2.9. An MDA-MB-468 subcutaneous breast tumor-bearing mouse was administered intravenously with a PMLA conjugate (Polycefin). 24 hours later, the animal showed drug distribution mostly in the tumor, as well as in kidney and liver (drug clearing organs).[63]

2.2.7. Critical aspects of PMLA

Today, up to my knowledge, there are other three groups working with PMLA, two in France (Dr. M. Vert and Dra. S. Cammas) which perform their research with synthetic PMLA; and one in the USA (Dra. J. Ljubimova with Dr. E. Holler) which works with biotechnological PMLA, who kindly provide us with biosynthetic PMLA. There are very few works dealing with PMLA modification and application in biomedicine, and moreover treating with biologically produced polymer; one of the main reasons is because it is not commercially available.

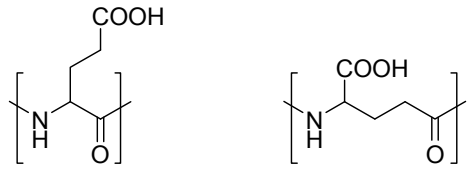
PMLA may be considered to a certain extent as a member of the PHAs family, which is wide-spread in many bacterial species, and it is available in relatively large quantities by fermentation. Production cost can be relatively low for PHAs, and some of the polyesters are eligible as raw materials for manufacturing plastics. In difference, PMLA is not competitive, since its production costs are still high and the biosynthesis has not been completely understood; furthermore, the high susceptibility of PMLA to hydrolysis makes difficult to obtain considerable amounts of material with an appropriate molecular weight, because prolonged

fermentation times results in the degradation of the all ready excreted polymer in the medium.[24] Same problem is expected for the post-polymerization modification, which must be done under soft conditions to avoid possible scission of the main chain.

PMLA is extremely water-soluble and therefore it is of complementary nature to PHAs which are water insoluble and limited to non-aqueous applications systems. Aside qualifying as raw material for the manufacture of water-soluble plastics or tissue, the polyanionic nature allows several other applications, some of which probably justify the relatively high productions costs. Those applications should be of value in the field of pharmacology, medicine and agriculture because ensures safe and healthy bioresorbability as it is a metabolite derived polymer. The main property that we explode in this work is the functionality of PMLA which makes possible the derivatization of the polymer, differentiating it from the most common and used biodegradable polymers in medicine. As more producing organisms are discovered, a diversity in structure and composition may become available that will render this material even more interesting.[24]

2.3. Biotechnological polymers: poly(γ-glutamic acid)

Poly(γ-glutamic acid) (PGGA) is one of the homo-poly(amino acid)s known to be present in nature produced by microorganisms;[64] it is a nylon 4 with a carboxylic group substitution pendant on the α-carbon. Poly(γ-glutamic acid) presents its peptide bond between the amino group of one monomer and the carboxylic group in γ position of the other monomer. It can be produced either by biological fermentation or by chemical synthesis. Like PMLA it has an stereoisomer, poly(α-glutamic acid) (Figure 2.10), which has been extensively studied by its biochemical incidence.



Poly(α, glutamic acid) Poly(γ-glutamic acid)

Figure 2.10. Chemical structure of poly(glutamic acid) stereoisomers.

PGGA natural presence was discovered in 1937 in the capside of *Bacillus anthracis*.[65] Later it was found to be the main product of the aerobic fermentation of *Bacillus subtilis*; but it was not until 1954 when Thorne and coworkers made the first research on PGGA biosynthesis and the optimization of the factors influencing biopolymer production.[66] Nevertheless the biochemical incidence of poly(α-glutamic acid), after some years of controversy now is known that biological PGGA is essentially constituted by γ bonds.[67] Regarding to its configuration, despite that the L-glutamic acid enantiomer is more abundant in nature, it is the D pair which predominates in PGGA biosynthesis, nevertheless its D:L enantiomeric composition varies largely depending upon the used bacterial strain and fermentations conditions.[68] In the decade of the 70s, Murao and coworkers studied PGGA production by *Bacillus subtilis* E5 on a culture medium with glucose and glutamic acid; they found that PGGA yields were higher than the quantities of acid administrated, so later they found that PGGA could be produced on a media only with urea and glucose.[69] Since then several works have been conducted to understand the biosynthetic pathways to increase PGGA productivity by different bacterial strains.[70-72]

In the same way as PMLA, PGGA can be chemically synthesized, either by polycondensation (Figure 2.11a) or by ROP (Figure 2.11b). First chemical synthesis was done by glutamic acid polycondensation, being necessary the α-carboxyl protection and γ-carboxyl activation and the preparation of the proper dimer to avoid undesired secondary reactions.

Polymer obtained by this method can be D or L homopolymers as well as stereocopolymers D/L, depending on the initial glutamic acid configuration; nevertheless these polymers resulted with low molecular weights.[73] With ROP technique, good yields and controlled molecular weights can be obtained from 3-(2,5-dioxo-1,3-oxazolidin-4-il)propionic acid a glutamic acid derivative. However, this synthetic route can be complicated because of the γ-N-carboxyanhydride instability which is formed during the process; thus molecular weight only reaches a few thousands because the decomposition products can limit the molecular weight.[74]

In difference with nylon 4, which is a conventional polyamide, PGGA is a highly hydrophilic polyamide due to its lateral carboxylic group with properties characteristic of a degradable material because of its susceptibility to hydrolysis in acidic and basic conditions. It is a polyacid with a pK_a ~2.27 that ionizes at increasing pH, considered as well as a polyelectrolyte,[75] it can be obtained in its acid or salt form depending on the ionization state. PGGA is obtained as a white semi-crystalline powder polymer but it melts with decomposition, so can be classified as a thermostable polymer with a melting temperature around 210 °C. Also, it is water soluble and its solubility strongly depends on the ionization degree and in its secondary structure. It is able to form α-helix and β-sheets; in the last case, the formation of hydrogen bonds limits its solubility. As a polyamide, it is susceptible to chemical hydrolysis, because the lateral carboxylic group increases its hydrophilicity and the water access to the polymer main chain. Degradation in water at ambient temperature is important, it increases notably with higher temperature and is accelerated under both acid and basic conditions.[76]

The γ peptide bond of PGGA differentiates it from proteins which have a peptide linkages formed between the α-amino and the α-carboxylic acid groups; the pendant free carboxylic group produces an asymmetric carbon in the structure and this functionality makes its derivatization feasible so the overall hydrophobicity of the polymer

could be modulated,[77] bioactive ligands can be introduced [78,79] or might associate with drugs;[80,81] thus PGGA has a countless number of possibilities without parallel in the field of conventional polyamides.[82] PGGA and its derivatives are considered promising biocompatible materials that could display functional properties of biomedical interest like drug delivery systems.

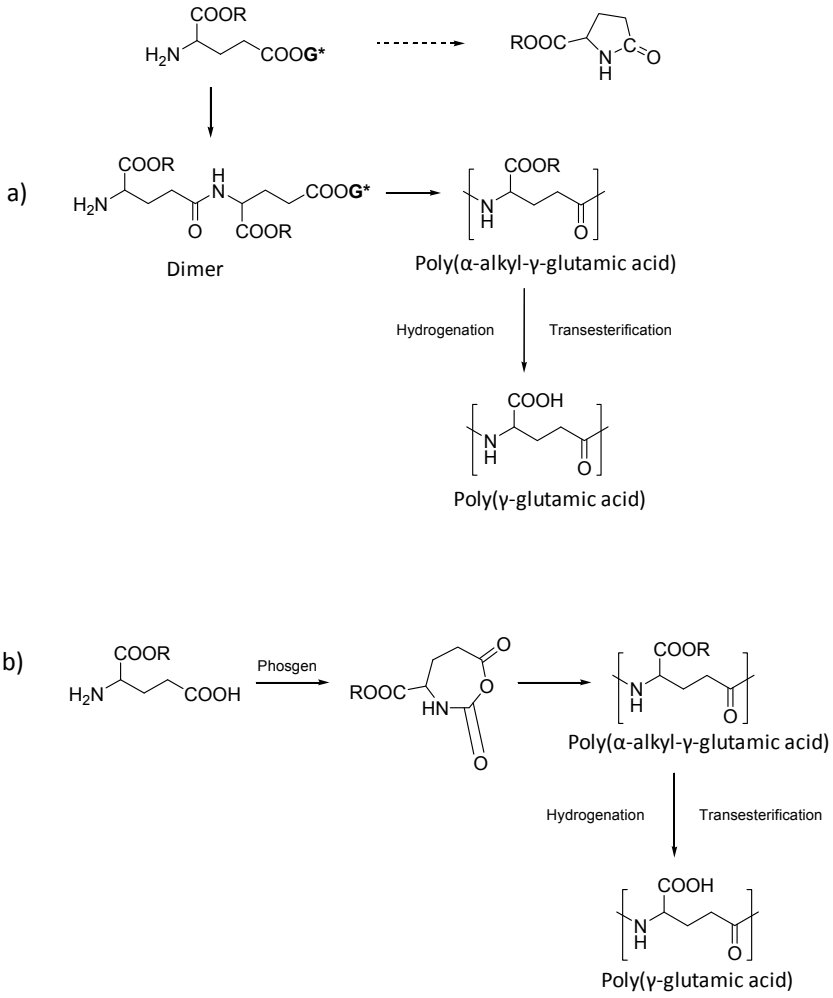


Figure 2.11. Chemical synthesis of poly(α-alkyl-γ-glutamate): a) poly-condensation. G* activated carboxylic group; and b) ring opening polymerization.

PGGA is object of current interest because of its natural origin, biodegradability, biocompatibility and bioresorbability, since it degrades into glutamic acid which is an essential substance to human.[82] PGGA biodegradation, as well as PMLA, depends in most part to enzymatic activity. Glutamic acid as a natural occurring amino acid is perfectly assimilable by the body. PGGA is edible and due to its biological properties as non-toxic and non-immunogenic material it is proper for biomedical applications as a drug delivery system,[81] bioadhesives,[83] material for tissue engineering [84] and hydrogels.[85] Several authors have investigated PGGA and its copolymers use in DDS for protein encapsulation,[86] cancer treatment,[81] as inmunoestimulant against viral and tumoral infections[87] among others; nevertheless, biomedical applications of PGGA remain as potential and poor studied. Up to day, as classified as GRAS (Generally Regarded As Safe) by the US Food and Drug Administration the main application of PGGA is in the food industry as flocculant and gelling compound.[88]

2.4. Polymer derivatization

Developments of original devices in the biomedical field are nowadays based on the use of biopolymers or biodegradable synthetic polymers answering to very strict conditions of applications. This has stimulated the modification of existing polymers or the development of new ones.[21] Moreover, current advances in the understanding of cellular and molecular biology have provoked an increasing need for materials with specific and better defined structures or functionalities; however, the preparation of such materials imposes major synthetic challenges.[89] In the drug delivery field, it should be noted that distinct mechanisms of drug controlled release require different polymers with a variety of physiochemical properties. Most of the polymers used in the development of DDS are designed with the capacity to form

supramolecular structures (matrices or capsules), which are suitable to retain therapeutic agents and deliver them under certain conditions.[8]

PMLA and PGGA have a great advantage for modification over the most used biodegradable polymers in biomedicine, *i.e.* PLA and PGA; because their inherent functionality that provides them very specific characteristics like their high hydrophilicity, and in consequence their susceptibility to hydrolysis. This functionality had caused great interest for the synthesis of new derivatives from these polymers which present better stability to degradation or show other specific functionalities, so the field of applications can be increased. But, at the same time, hydrolysis susceptibility restricts the modification methods that can be applied for direct derivatization. With the exception of certain ionic coupling reactions that have been recently reported,[90-92] most of the chemical modifications carried out on the carboxylic side group involve the covalent attachment of organic compounds.[51,53,58,77,93] The most common modification is the lateral carboxylic group esterification or amidation, which is used with the aim of modulate their hydrophobicity in accordance to the type, conversion degree and substitution distributions or to introduce bioactive ligands that will give specific properties to the polymer.

With respect to biomedical applications, hydrophobic character modulation is of great importance; as more hydrophobic is the conjugate, easier is the penetration into the cell by membranolysis.[46] Also it has been shown that hydrophobization of PMLA conjugates carriers promoted cell penetration.[18] In the case of brain tumors treatment, it is necessary that the systems be able to penetrate de blood brain barrier (BBB). This membrane is extremely permioselective, avoiding the penetration of hydrophilic compounds that could be in the blood stream and that can damage the brain, and in the same way is a limiting barrier for DDSs.

The carboxylic side groups of the polyacids may serve as excellent points to build graft copolymers by either attaching polymeric side chains (grafting-on) or initiating the growth of polymeric branches (grafting-from).[82] In addition to the random copolymers formed by direct modification of the pendant carboxylic groups, block- copolymers containing long homogeneous sequences of PMLA or PGGA linked to other homopolymeric segments of different properties have been developed by synthetic methods for their self-assembling properties and potential application in biomedicine.[53] A terpolymer from PMLA derivatives units have been prepared by ROP to mimic heparin sulfates in order to enhance bond and tissue repair.[45]

The most advanced case for application of a PMLA derivative is Polycefin, a multifunctional nanoconjugated that use PMLA as a backbone. The ability of Polycefin to go through the blood brain barrier depends on the hydrophobic character of the molecule, which is determined by the different nature of the grafted moieties;[50] also a tandem of recognition molecules have been added for an efficient targeting delivery,[94] and with capability for carrying chemotherapeutic agents[62] or gene therapy (Figure 2.12).[50] These have been achieved by the activation of the carboxyl groups and the posterior grafting of the different functional moieties.

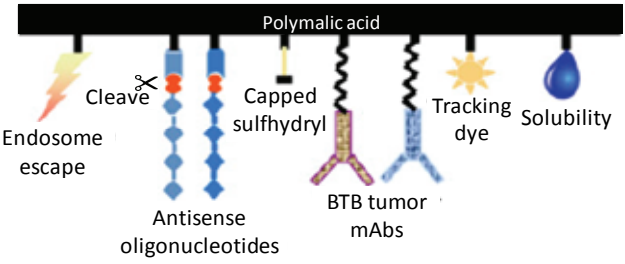


Figure 2.12. Cartoon of PMLA-based multifunctional nanoconjugate: Polycefin.[59]

2.4.1. Esterification

PMLA modification presents a big challenge because the labile nature of the main chain ester bond; this make mandatory to perform specific reactions under smooth conditions if the molecular weight is to be conserved. Thus, condensation methods for esterification are practically unviable because of the main chain scission that occurs during this process. For this reason, most of the PMLA derivatives have been obtained by chemical synthesis (ROP) with the appropriate monomers.[5,53] Recently in our group, Fernandez and coworkers made the esterification of biologically produced PMLA with diazomethane (Figure 2.13) obtaining poly(α-methyl β,L-malate) 100% esterified without reduction in the molecular weight, but this method is limited to methyl derivatives.[51] The same method was used for the production of fully and partially methylated PMLA for the production of micro- and nano- spheres for antibiotic and proteins encapsulation, respectively.[48,95]

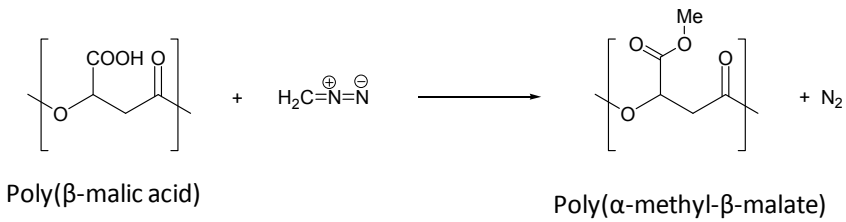


Figure 2.13. PMLA methyl esterification with diazomethane.

In the case of PGGA other esterification methodologies can be applied since the amide bond in the main chain confers more stability to more aggressive reaction conditions. The first and most frequent method for PGGA esterification is based on the reaction with alkyl bromides in the presence of sodium hydrogen carbonate using an organic solvent as DMSO or NMP (Figure 2.14).[96] This methodology has been extensively

applied by different authors who introduced more or less important modifications in the reaction conditions in order to improve yields and conversions.[77,97] In recent years, a new procedure for the esterification of PGGA has been developed; which consist of two steps; first, ethylation with ethyl bromide followed by transesterification with alcohols in the presence of titanium tetrabutoxide.[98]

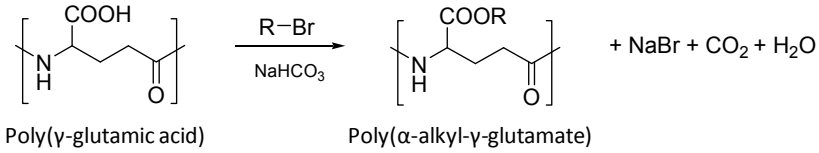


Figure 2.14. PGGA esterification with alkyl bromides.

2.4.2. Amidation

In addition to the esterification reactions, amidation is another option for direct modification on the polycarboxilates like PMLA and PGGA. PMLA amidation has been done in the development of Polycefin, it has been achieved by the activation of the carboxylic groups by N-hydroxysuccinimide and a posteriori amidation by nucleophylic attack by primary amines on the activated polymer (Figure 2.15). By this technique PMLA has been amidated with NH_2-poly(ethylen glycol), membrane disrupting peptides, drugs attached through pH sensitive hidrazide linkages, antibody targeting molecules and fluorescent probes.[50]

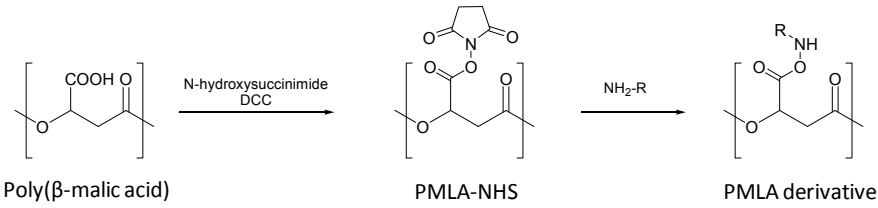


Figure 2.15. PMLA amidation through carboxyl group activation with N-hydroxysuccinimide.

The carboxylic group of PGGA is susceptible to being amidated with a variety of amino compounds; reaction is assisted by a convenient activating agent such as a carbodiimide. Although different amino compounds have been covalently attached to PGGA, conjugation with naturally occurring amino acids is among preferred grafting approach. Nontoxic hydrophobic esters of amino acids, in particular the ethyl ester of L-phenylalanine, have been extensively explored for producing amphiphilic PGGA derivatives able to self-organize in structured nanoparticles. The degree of amidation determines the type of molecular association that operates in the building of the particle and is therefore critical in establishing its size.[84]

2.4.3. Ionic coupling

The capacity of polyelectrolytes to form more or less stable complexes upon coupling with opposite charged ionic compounds is well known. In the case of the counter ion has a noticeable hydrophobicity, the water solubility of the original polyelectrolyte is lost and the complexes become soluble in organic solvents. Furthermore, the complexes usually display a pattern of physical properties largely dissimilar to those of the parent polyelectrolyte, which is in many cases the objective of their preparation.

PMLA and PGGA are able to form stoichiometric ionic complexes with alkyltrimethylammonium surfactants and with a precise composition and satisfactory stability creating comb-like nanostructured polymers.[90,91,99] As polyelectrolytes, these macromolecules are sensible to pH, this character has been used for pH responsive systems in the biomedical field. An example of the application of PGGA to the design of pH sensitive DDS is the production of nanoparticles through the ionic interaction of the negative charged PGGA with the polycation chitosan for encapsulation of heparin, a fibroblast grow factor, with a pH dependent release[100] or the PGGA complexation with DOX and a cationic lipid which results in nanometric particles with potential for targeted delivery in solid tumors.[101]

A different strategy that could bring much interest because of its simplicity is the direct ionic complex formation between a polyion and an opposite charge ionic drug; this strategy has been directed for drug-loading on polymers more than for modification. The simplicity and interest of this type of complexes reside in the fact that they are formed in aqueous solutions without the need of organic solvents and without secondary products. A ionic complex between polyaspartic acid and the cationic drug diminazene, a model drug, had been done, the study revealed that ionic interactions and hydrogen bonds were present.[102] PGGA ionic complex with the cationic drug Doxorubicin, a cancer chemotherapeutic, had been also studied; this complexation leads to the formation of nanoparticles which shows a pH dependent drug release.[103]

2.4.4. Thiol-ene click reaction

To address the gap between the sophisticated functionality that is required for future advances in bio- and nano- technology and the limited chemical control offered by many of the synthetic processes that are currently available, we are now witnessing an increasing application

of synthetic organic chemistry concepts into material science.[89] The concept of postpolymerization functionalization strategies introduces a number of major challenges, such as efficiency and orthogonality, which must be overcome.[104]

An excellent example of the power of postpolymerization strategies is the wide range of functional materials that have been prepared using Cu-catalyzed azide-alkyne *click* chemistry; but for biomedical applications an alternative without metallic catalyst is preferred, like thiol-ene reactions (Figure 2.16).[105] *Click* chemistry has been shown to be synthetically efficient,[106] specifically, radical-mediated thiol-ene reaction as a facile and convenient tool for the postpolymerization modification of well-defined reactive polymers and for the construction of complex macromolecules. While only recently recognized and exploited as a *click* process, the thiol-ene reaction, in both, its radical and base/nucleophilic forms, has already been demonstrated to be a powerful and versatile method for site specific functionalization and as a convenient conjugation tool.[107] There are several features associated with the thiol-ene reaction that make it a particularly attractive, facile and versatile process; the reactions must: *i*) result in a stable linkage, *ii*) exhibit minimal cross-reactivity with other functional groups, *iii*) react to completion, *iv*) be free of appreciable amounts of side products, and *v*) proceed under benign reaction conditions.[108] *Click* chemistry provides alternatives to conventional strategies used for postmodification of side chains for PGGA esters; a two step modification is possible under the mild conditions used in modern *click* conjugation strategies.[88,109] Furthermore, the vast array of commercially available molecules and biomolecules with either thiol or ene functionality makes this a powerful technique to introduce functional groups.

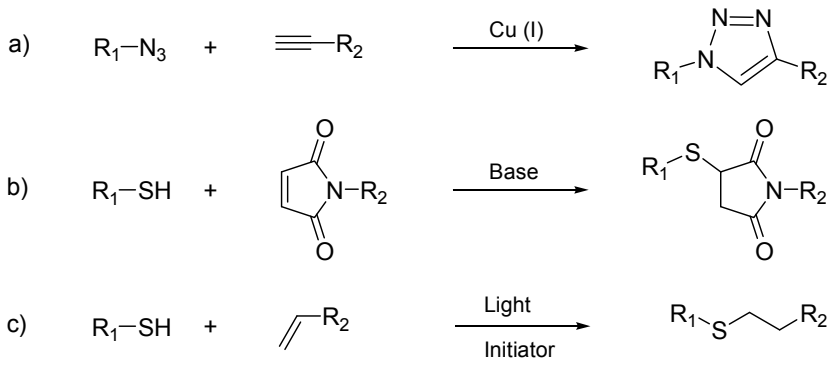


Figure 2.16. *Click* reaction forms: a) copper catalized azide-alkyne, b) base-catalyzed thiol-ene and c) radical photoinitiated thiol-ene.

Thiol-ene photopolymerization for biomedical applications was recently presented as a novel mechanism for cross-linking and hydrogel formation. This technique has been exploited to fabricate protein delivery vehicles capable of enzyme-responsive drug release.[110] Also, thiol-ene reactions had been used for the stabilization and functionalization of polymer multilayer-coated particles and capsules for a new generation of particulate delivery systems and microreactors.[111] For functionalization, covalent attachment of biomolecules to polymers have been performed like the site-selective conjugation of peptides and proteins using the disulfide bridge when it is not essential for bioactivity retention,[112] or polymer functionalization with cysteamine which provides a spot for Doxorubicin conjugation onto the polymer through a pH-sensitive hydrazone bond.[113] For more complex architectures *click* reactions also were successfully applied for the synthesis of dendrimers as a possible drug delivery vehicle with the conjugation with cisplatin.[114]

2.5. Nanoparticles for drug delivery systems

Application of nanotechnology in medicine is known as nanomedicine and it is attempted to improve life quality of human beings, fighting against diseases in an innovative way. Most of the effectiveness of an oral drug is lost when it is metabolized by the leaver or digested in the digestive track. Pharmaceutics solution to this is to increase the drug amount in each tablet, taking in account how much is going to be lost during the administration. Employing different routes of administration, like intravenous or intramuscular injections, has resulted in a reduction of losses. The use of nanoparticles goes further because it allows the penetration of cell membranes to introduce chemical, biological or genetic therapeutic material in determined cells. This nanodelivery promises revolutionary treatments for incurable diseases up today.[115]

In the last decades, nanotechnology has focused on formulating therapeutic agents as biocompatible nanocomposites such as nanoparticles, nanocapsules, micellar systems and conjugates (Figure 2.17); were drugs are entrapped, encapsulated, dissolved or attached to a polymer matrix. Polymers able to delivery active agents are becoming more popular since they are less immunogenic than viral vectors[50] and offer some advantages over liposomes, like better stability protection for drugs and proteins and better properties for controlled release.[6] For these, a great variety of polymers have been tested for DDS and they can effectively deliver a drug to a target site increasing the therapeutic benefit, while minimizing toxicity and side effects; but it is biodegradable nanoparticles which have taken great attention as effective carrying devices, because they possess useful controlled release properties and do not accumulate in the body.[116]

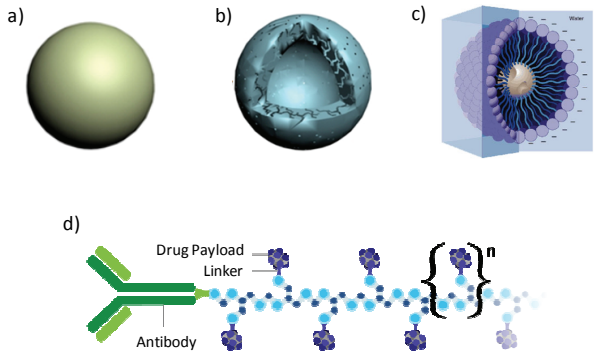


Figure 2.17. Types of drug delivery systems: a) particles, b) capsules, c) micelles and d) conjugates.

DDS systems based on polymer particles, either nanoparticles or microparticles, are clearly advantageous because: *a)* particle size and surface can be engineered to achieve passive or active drug targeting, *b)* drugs can be incorporated without chemical reaction, *c)* drug activity is optimally preserved during its transportation to the site of action, *d)* site-specific targeting can be achieved by attaching targeting ligands, *e)* formulation can be delivered through different routes of administration, *f*) toxicity of active compounds is reduced by controlling its delivery *g)* controlled and sustained drug release can be achieved, and *h)* particle degradation can be modulated by polymer matrix choose.[2,6,80,116,117]

Microparticles were among the first controlled delivery systems, administration could be subcutaneous, intramuscular, or intraperitonial; but because of their size they are not able to circulate through the capillary vessels. To overcome this, nanoparticles are now the focus of research, they are defined as particulate dispersion or solid particles with a size in the range of 10-1000 nm and they offer several advantages over microparticles like deep penetration into tissues through capilars, pass through the epithelial membrane and better uptake by cells.[118] Regardless, nanoparticles do have limitations; for example, their small

size and large surface area can lead to particle aggregation, making physical handling difficult; in addition, same factor results in limited drug loading, an initial burst release and the recognition and fast clearness by the body immune system.[116]

Nanoparticles in medicine have been used not only as a DDS, but also for fluorescence, magnetic resonance and X-ray imaging. Nevertheless, most of the works deals with drug delivery, especially in cancer therapy. In chemotherapy DDS has spread out rapidly because their use can reduce systemic toxicity and minimize the side effects on normal cells while drug concentration at specific sites increases by administering the drug directly to cancer cells; besides, some systems offer the possibility of crossing the blood brain barrier, a permoselective membrane that protects the brain. For example, one of the main challenges in cancer chemotherapy is drug formulation, which normally involves toxic excipients. Nanoparticle-based DDS provide an alternative over the use of toxic adjuvants by encapsulating the drug.[80] Non-toxic drug formulations must be used for the treatment of tumor cells because it is toxicity of normal cells that constrains dose and frequency, both important factors in cancer chemotherapy treatment.[119]

To achieve an efficient DDS we must to consider the physicochemical properties of the main three components involved in the DDS application; the vehicle (i.e. polymer matrix), the active compound to be delivered and the target compartment (organ or cells). As it is not possible to modify the properties of the target compartment and limited in the modification of the active compound, the challenge is to determine the optimal physicochemical properties of the polymer to confer drug loading efficiency, long circulation times, site recognition targeting and controlled drug release.

Conventionally, nanoparticle preparation methods could be split in two mayor classes: *i*) from an existing polymer; and *ii*) during polymerization process. In the first case, several methods have been

proposed for dispersing preformed polymers like PLA, PGA, PLGA and PC: emulsion-solvent evaporation, spontaneous emulsification/solvent diffusion, salting out/emulsification-diffusion, adaptations of these methods with the use of supercritical fluids, nanoprecipitation, ionic gelation, coacervation of hydrophilic polymers and spray drying.[116,120] Through the 70s and 80s, most degradable drug-loaded microparticles were formed either by solvent evaporation from emulsion or by phase separation techniques.[2]

2.5.1. Emulsion solvent evaporation technique

Microparticles and nanoparticles can be formed by emulsion solvent evaporation method, it have been widely applied in the last decades either with synthetic or natural polymers for the preparation of a large diversity of formulations with different applications, from biomedical science to the textile and shoe industry.

The solvent evaporation method is a two-step process; first, the polymer is dissolved in an organic solvent like dichloromethane, chloroform or ethyl acetate, the solution is then emulsified into an aqueous solution of a surfactant (*i.e.* gelatin, PVA, polysorbate-80) to form an oil in water emulsion (O/W) by shear stress produced either by agitation, homogenization or sonication. Second step, after the formation of a stable emulsion, is solvent removal which can be achieved by evaporation or liquid extraction; in the first case, volatile solvent from the polymer dispersion is removed by increasing the temperature or reducing the pressure under agitation; while the liquid extraction is done by the addition of continuous phase or additional extraction agents which absorb the entire solvent leaching from the solidifying particles (Figure 2.18).[120] This method is good for a laboratory scale, but for larger scales alternative methods are used.[6] For drug encapsulation the drug is

dispersed or dissolved into the preformed organic polymer solution before the emulsion formation.

Particle size and size distribution will be mainly determined by the size of the droplet of the emulsion disperse phase, which is determined by phases' viscosities, energy applied for emulsion generation, and interfacial interaction of both phases.[121,122] Fast solvent removal from emulsion also increased the control on particle size and distribution without physical aggregation.[123] Particle size may affect drug release rate, drug encapsulation efficiency, product syringeability, *in vivo* fate in terms of uptake by phagocytic cells and biodistribution of the particles after administration.[120,124]

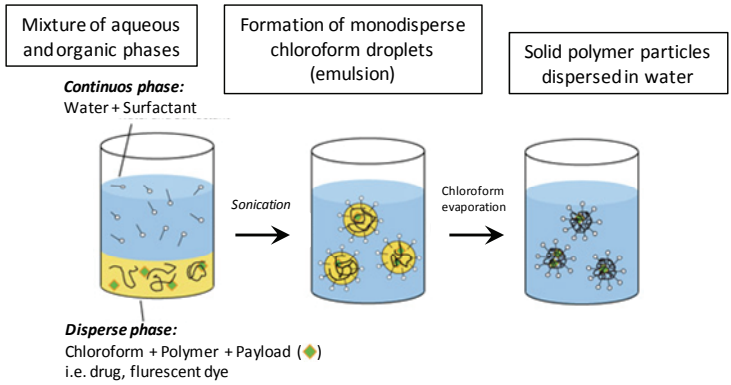


Figure 2.18. Particle formation by emulsion-solvent evaporation technique.[125]

To prevent coalescence of the drug/matrix dispersion droplets, a surface active or viscosity enhancer stabilizer as PVA is generally added to the continuous phase, drug encapsulation efficiency can be improved by controlling the molecular weight of PVA as surfactant.[123] Another emulsifying agent that is used for the production of particles by solvent evaporation is PEG which also renders to the nanoparticles mask characteristics against reticulo-endothelial system recognition.[126] Since

the emulsion stabilizer stays at the oil/water interface during solvent evaporation, there is a possibility of adsorption of the stabilizer; binding of surfactant on the particles may alter the physicochemical surface properties.[127]

Normally, the O/W emulsion is applied for hydrophobic molecules encapsulation, but there is a variation of this method which employs a double emulsion technique for the encapsulation of hydrophilic drugs. The drug is first dissolved in aqueous media (W_1) and emulsionated in an organic polymeric phase (O), the stable emulsion (W_1/O) is then emulsionated again in the aqueous continuous phase (W_2) forming a double emulsion system ($W_1/O/W_2$), which leads to particle formation after solvent removal.[124]

2.5.2. Nanoprecipitation: self-assembled structures

Self-assembly is an important driving force in the formation of a variety of highly ordered structures for polymeric macromolecules that are useful for different applications. The most commonly used interactions include electrostatic and hydrogen bonding, as well as amphiphilic forces.[128] Amphiphilic block or graft copolymers consisting of hydrophilic and hydrophobic segments are self assembling materials capable of forming polymeric associates in aqueous solutions due to the intra- or inter- molecular hydrophobic interactions.[129]

This method basically consists in the change of solubility conditions of the polymer solution, so the polymer will self-arrange in a defined structure depending on chemical structure and solubilization-precipitation conditions. Under selective solvent conditions, the copolymers adopt various organized structures; multimolecular spherical core-shell nanoparticles, having an inner-core made up by the insoluble blocks and an outer-shell built by the soluble blocks, represent

the most frequent structure and belong to the family of colloidal polymers (Figure 2.19).[53]

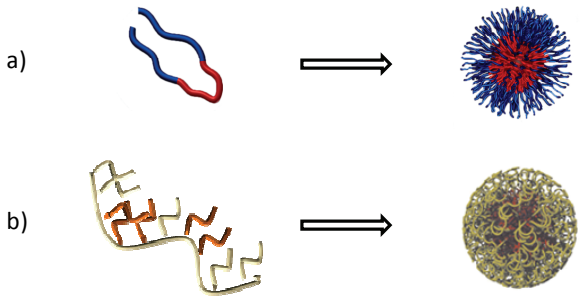


Figure 2.19. Self-assembled particles from amphiphilic polymers: a) block copolymer and b) graft copolymer.

The parameters which influence the self-assembly of molecules in solution include the temperature and solvent quality. Mixing two solvents is the most common and flexible way to achieve a large and rapid change in solvent quality. The critical attribute that drives micellization, such as solvent quality or temperature, should be rapidly passed to yield an initial supersaturation ratio. To impart colloidal stability and avoid fusion, the copolymers should contain a soluble portion of sufficient size for steric or electrostatic repulsion.[130]

Grafting of hydrophobic amino acids or peptides on water-soluble polymers has been done with the aim of giving an amphiphilic character to the polymer so it could form nanoparticles.[93] The hydrophobic inner core of the structure acts as an incorporation site for therapeutic agents, especially hydrophobic drugs. These systems can be used to provide targeted cellular delivery of drugs, to avoid toxic effects, and to protect the active compound against biodegradation.[84] Degradable macromolecular micelles based on amphiphilic synthetic block copolymers of PMLA and PMLA alkyl esters have been produced for

drug carriers[53] and also nanoparticles form amphiphilic graft copolymers by partial methylation of biological PMLA for protein encapsulation and delivery[95] or partial esterification with alkyl chains of PGGA for erythromycin and protein delivery.[86] The success of most advanced drug delivery strategies requires development of sophisticated new site-specific carriers. Thermoresponsive[131] and pH-sensitive[113,132] micellar DDS have been developed form block and graft copolymers by dialysis techniques. An advantage of the precipitation dialysis method over emulsion evaporation is the elimination of the use of high stress devices like sonicators or homogenizers avoiding potential damage.[133]

2.5.3. Drug encapsulation and release

Micro- and nano- particles are systems of great interest as drug delivery systems of high and low molecular weight active compounds. Biopolymer particles-based drug delivery systems provide an ideal alternative for drug encapsulation since the activity, solubility, cell permeability, and stability of drugs can be manipulated by using polymers with different chemical and physical properties;[103] being able to convert poorly soluble, poorly absorbable and biologically labile active substances into promising deliverable drugs.

Drug can be dissolved, dispersed, encapsulated or attached to the polymeric matrix; and depending upon the preparation method, nanoparticles or nanocapsules can be obtained. Nanocapsules are vesicular systems in which the drug is confined into a cavity surrounded by a polymeric membrane; while nanoparticles are matrix systems in which the drug is uniformly dispersed.[6] Covalent bonds used for drug-polymer conjugation can be pH[79,134,135] or Redox[136] sensitive to address a site specific delivery. Nevertheless, active compounds can also be linked through non-covalent interactions like hydrogen bonds or ionic and hydrophobic interactions.[103,137] But in general, like for cancer treatment,

free drug release from the polymer is a prerequisite for the activity of most of the active agents. To achieve an effective drug release and therapeutic effect a proper choice of the type of linkage between drug and carrier is relevant[138] so the association between both components could be broken for a successful time- or site- delivery.

The literature describes three main mechanisms for drug release from polymeric particles systems, which primarily differ in the role that the carrier plays in controlling the release. One is controlled release by swelling; in which polymer hydration causes a volume increase of the polymeric structure and the resulted pore size increase allows diffusion of the aqueous medium within the polymeric matrix and thus the drug release. Another mechanism is by polymer degradation; hydration of the polymer results in hydrolytic degradation with release of its contents, release and degradation depends on the stability of the polymer linkages. And finally, there is drug release by pure diffusion; where the compound diffuses through the voids of the polymeric device (Figure 2.20).[8] If the diffusion of the drug is faster than matrix degradation, then the mechanism of drug release occurs mainly by diffusion. However, in biodegradable polymers release generally occurs by a combination of the three mechanisms. The characteristics of each particle differ in terms of drug loading capacity, particle and drug stability, drug release rates, and targeted delivery ability.[119]

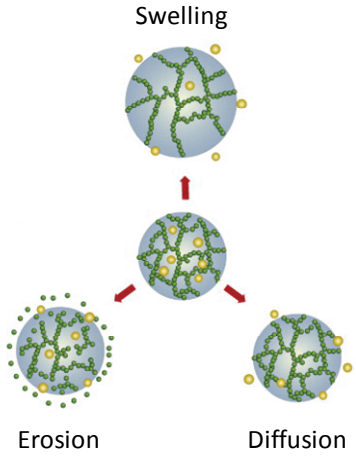


Figure 2.20. Drug release mechanisms from polymeric particulated delivery systems.[139]

3

Materials and Methods

3.1. Materials

Poly(β,L-malic acid) (PMLA) sample used in this work was provided by Dr. Portilla (Cedars-Sinai, Los Angeles, USA). It was biotechnologically produced by aerobic cultivation of *Physarum polycephalum* and isolated and purified as described elsewhere.[24] Purity of the sample was ascertained by 300 MHz ^{1}H NMR, and it had a weight-averaged molecular weight between 25-34 kDa with a dispersity of 1.08-1.2 as determined by GPC. PAALM-1 was prepared by methylation of PMLA with diazomethane in dry acetone as previously described in detail.[51] Poly(γ,glutamic acid) (PGGA) sample used in this work was kindly supplied by Dr. Kubota of Meji Co. (Japan). It was produced by fermentation of *Bacillus licheniformis*, with a weight-average molecular weight approximately of 30 kDa and a nearly racemic composition. L-Leucine ethyl ester, L-phenylalanine methyl ester, allyl alcohol, allyl bromide, 1-octanothiol, 1-dodecanothiol, 1-hexadecanothiol, 2,2-dimethoxy-2-phenylacetophenone (DMPA), dicyclocarbodiimide (DCC) and Theophylline (1,3-dimethylxanthine) (TEO) were purchased from Sigma-Aldrich. Temozolomide (3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-as-tetrazine-8-carboxamide) (TMZ); Doxorubicin ((7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione) (DOX) and Carbamazepine (5H-dibenz[b,f]azepine-5-

carboxamide) (CBZ) were obtained from AKSci (Union City, CA, USA). All organic solvents were either analytical or HPLC grade and they were used without further purification.

3.2. Synthesis of poly(β,L-malic acid) derivatives

The synthesis of each derivative is presented in the corresponding chapter; Table 3.1 shows the synthesized PMLA derivatives, their acronyms and the chapter number of the correspondent results and discussions.

3.3. Hydrolitic degradation

Hydrolytic degradation rate of PMLA derivatives was evaluated by following the change in molecular weight as function of time from samples incubated in aqueous buffers. For this, series of polymer nanoparticle or powder samples of about 2 mg each were immersed in citrate buffer pH 5.0 or phosphate buffer pH 7.4 at 37 °C, and collected at scheduled times for GPC analysis.

For the assessment of the hydrolytic degradation mechanism, 10 mg of polymer were placed in NMR tubes containing 1 mL of deuterated water or buffered solution, incubated at 60 °C and analyzed by ^{1}H NMR at scheduled times; soluble degradation products released to the incubation medium were identified and their relative amounts monitored at different time points.

Table 3.1. Synthesized derivatives.

Acronym	Derivative	Chapter
PAALM-1	poly(α-methyl-β,L-malate)	4.
PMLA-Et_{100}	poly(α-ethyl-β,L-malate)	5.
co PMLA-$Et_{50}H_{50}$	*co*poly(α-ethyl-β,L-malate-*co*-malic acid)	
PMLA-Bu_{100}	poly(α-butyl-β,L-malate)	
co PMLA-$Bu_{50}H_{50}$	*co*poly(α-butyl-β,L-malate-*co*-malic acid)	
PAALM-L_x	*co*poly(α-leucine ethyl ester-β,L-malate-*co*-malic acid)	6.
PAALM-F_x	*co*poly(α-phenyalanine methyl ester-β,L-malate-*co*-malic acid)	
PMLA/DOX	poly(β,L-malic acid)/Doxorubicin ionic complex	7.
*co*PMLA-$PrSR_xH_y$	*co*poly(α-3-alkylthio-porpyl-β,L-malate-*co*-malic acid)	8.
*co*PGGA- $PrSR_xH_y$	*co*poly(α-3-alkylthio-porpyl-γ,D-glutamate-*co*-glutamic acid)	

3.4. Nanoparticles preparation and drug encapsulation

Two methods were employed for nanoparticles formation depending upon the modification degree of the polymer. For 100 % modified polymers (PAALM-1, PMLA-Et_{100}, PMLA-Bu_{100}), which were hydrophobic polymers soluble in dicloromethane (DCM), the emulsion solvent evaporation method was applied. Briefly, 20 mg of polymer were dissolved in 1 mL of DCM, added to 10 mL of 0.2-2 % PVA (M_w~2000/14000) aqueous solution (Table 3.2) and emulsified with a tip probe sonicator (Bandelin, Berlin, Germany, Sonoplus, 200W) operating with intermittent pulses at 50 % of amplitude during 45 seconds. Emulsion was then dispersed in 20 mL of water and DCM was evaporated under reduced pressure in a rotary evaporator. Nanoparticles were recovered from the aqueous suspension by centrifugation; washed 3 times with distilled water to eliminate the emulsifier excess and freeze-dried for storage.

The precipitation dialysis method was applied for nanoparticle formation when partially modified PMLA were used (PAALM-L*x*, PAALM-F*x*, *co*PMLA-$Et_{50}H_{50}$, *co*PMLA-$Bu_{50}H_{50}$, *co*PMLA-$PrSR_xH_y$ and *co*PGGA-$PrSR_xH_y$). In this case, to a solution of 2.5-10 $mg \cdot mL^{-1}$ of copolymer in DMSO, NMP, acetone or methanol (Table 3.3), 1 mL of water was added dropwise under magnetic stirring. The mixture was dialyzed against distilled water for 24 h using a cellulose membrane with a molecular weight cut-off of 8 kDa. NPs formed inside the bag were recovered by freeze-drying. Particle morphology was monitored by scanning electron microscopy (SEM) and their average hydrodynamic diameters and surface charge were determined by dynamic light scattering (DLS) and ζ-potential measurements, respectively.

Table 3.2. Conditions for nanoparticle formation and drug encapsulation with the emulsion solvent evaporation method.

Derivative	[Polymer solution][a]	Surfactant	[Surfactant][b]	Drug[c]	$[Drug]_i$[d]	Chapter
PAALM-1	2 %	PVA 2 kDa, PVA 14 kDa, PMLA 34 kDa, none	0.2, 0.5, 1, 2 %	TMZ, DOX	10 %	4.
$PMLA\text{-}Et_{100}$ $PMLA\text{-}Bu_{100}$	2 %	PVA 2 kDa	1 %	TMZ, DOX	10 %	5.

[a] Polymer solution concentration: % (w/v)
[b] Surfactant solution concentration: % (w/v)
[c] Encapsulated drug.
[d] Drug concentration in the organic solution related to the polymer (w/w).

For drug loading, 10-30 % (w/w) of drug was added to the initial organic polymer solution used either in the emulsion solvent evaporation or in the precipitation dialysis method (Tables 3.2 and 3.3). In the case of DOX addition of triethylamine (TEA) previous to emulsification was necessary to render the drug solubilized in DCM in the emulsion solvent evaporation method. In the case of PAALM-L and PAALM-F derivatives, methanol was used as cosolvent for drug encapsulation and was removed by rotaevaporation to avoid drug losses during dialysis. Precipitation dialysis procedure was used for TEO and CBZ encapsulation but using drug-saturated water for the dialysis process to reduce losses during dialysis.

Table 3.3. Conditions for nanoparticle formation and drug encapsulation with the precipitation-dialysis method.

Derivative	Solvent	[Poly][a]	Drug[b]	[Drug]$_i$[c]	Chapter
*co*PMLA-Et$_{50}$H$_{50}$ *co*PMLA-Bu$_{50}$H$_{50}$	DMSO	1 %	TMZ, DOX	10 %	5.
*co*PAALM-L$_x$ *co*PAALM-F$_x$	DMSO, Methanol, Acetone	0.5, 1 %	TMZ, DOX	30 %	6.
*co*PMLA-PrSR$_x$H$_y$	DMSO	0.25 %	TEO, CBZ	20 %	8.
*co*PGGA-PrSR$_x$H$_y$	NMP	1 %			

[a] Polymer concentration: % (w/v).
[b] Encapsulated drug.
[c] Drug concentration in the organic solution related to the polymer (w/w).

Drug content was determined by dissolving 5 mg of drug-loaded nanoparticles either in DMSO, methanol or DCM and quantifying drug concentration by UV-vis spectrophotometry. Drug concentration was calculated from a calibration curve using known amounts of free drug as standards. Absorbance of drugs was measured at the following wave lengths: TMZ (330 nm), DOX (480 nm), TEO (275 nm) and CBZ (220 nm).

Encapsulation efficiency (EE) was calculated on the basis of the following formula:

$$\% \text{ EE} = \frac{[\text{Drug}]_{final}}{[\text{Drug}]_{initial}} \cdot 100 ,$$

where $[Drug]_{final}$ = drug concentration in the nanoparticles (w/w) and $[Drug]_{initial}$ = drug concentration in the initial organic solution related to the content of polymer (w/w).

3.5. Drug loaded films

Drug-loaded films (*co*PMLA-PrSR$_x$H$_y$ and *co*PGGA-PrSR$_x$H$_y$) were prepared by casting. Briefly, 1 mL of 2 % polymer solution in $CHCl_3$ and 0.5 mL of 0.4 % CBZ solution in $CHCl_3$ were mixed and slowly evaporated on a Teflon cast (20 % w/w CBZ/polymer). TEO loaded films were not possible to make because there was not a common solvent for polymers and drug. DSC analysis was performed on drug-loaded films to determine the crystalline state of the drug in the films.

3.6. In vitro drug release

In vitro drug release was evaluated by the dialysis method. Briefly, 10 mg of freeze-dried drug-loaded nanoparticles were resuspended in 1 mL of citrate buffer at pH 5.0 or phosphate buffer either pH 6.8 or 7.4 (Table 3.4), and transferred into a dialysis tube with 8 kDa molecular weight cut-off. The tube was then immersed into 20 mL of buffer and left at 37 °C under slight stirring. 0.5 mL aliquots of the releasing medium were taken at scheduled times and the drawn volume replaced by fresh buffer. Drug concentration was determined by high performance liquid

chromatography (HPLC) at 330 and 480 nm for TMZ and DOX, respectively, using known amounts of free drugs as standards. Since TMZ is hydrolytically labile, its degradation product 5-aminoimidazole-4-carboxamide (AIC) absorbing at 254 nm was also accounted.

In vitro DOX release from PMLA/DOX conjugates was evaluated also as function of pH and ionic strength. Briefly, 10 mg of freeze-dried PMLA/DOX ionic complex particles were resuspended in 1 mL of buffer at either pH 5.0, 150 mM NaCl ionic strength, and at pH 7.4, 75, 150 or 300 mM NaCl ionic strengths (Table 3.4). 2 mL aliquots of the releasing medium were taken at scheduled times and the drawn volume replaced by fresh buffer. Drug concentration was determined by UV-vis spectroscopy at 480 nm and cumulative drug release was calculated as a function of time.

Theophylline and Carbamazepine concentrations were also determined by UV spectroscopy at 220 and 275 nm for CBZ and TEO, respectively. Cumulative drug release was calculated as a function of time.

Table 3.4. Buffer conditions for *in vitro* drug release assays.

Derivative	Buffer	pH	Ionic strength
PAALM-1	Phosphate	6.8, 7.4	
PMLA-Et_{100} *co* PMLA-$Et_{50}H_{50}$ PMLA-Bu_{100} *co* PMLA-$Bu_{50}H_{50}$	Phosphate	7.4	
*co*PAALM-L_x *co*PAALM-F_x	Phosphate	7.4	
PMLA/DOX-	Citrate Phosphate	5.0 7.4	150 mM 75, 150, 300 mM
*co*PMLA-PrSRxHy *co*PGGA-PrSRxHy	Phosphate	7.4	

3.7. Cell lines and culture media

Cell lines used in citotoxicity studies were primary human glioma U-87 MG and T98G, human invasive breast carcinoma MDA-MB-231 and MDA-MB-468, and human brain metastatic lung cancer CRL-5904, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). U-87 MG and T98G cells were cultured in MEM media supplemented with the following ingredients (final concentrations): 10 % fetal bovine serum, 1 % MEM NEAA, 1 mM sodium pyruvate and 2 mM L-glutamine. For MDA-MB-231 and MDA-MB-468, Leibovitz's L-15 medium with 10 % final concentration fetal bovine serum was used, and for CRL-5904, RPMI 1640 (ATCC) medium was used. Cells were seeded at 10^4 cells per well (0.1 mL) in 96-well flat-bottomed plates and incubated overnight at 37 °C in humid atmosphere with 5 % CO_2 for U-87 MG, T98G and CRL-5904 and without CO_2 for MDA-MB-231 and MDA-MB-468 cell lines.

3.8. Cytotoxicity tests and nanoparticles cellular uptake

Cytotoxicity studies were performed on cells incubated for 1, 12, 24 and 72 hours with freshly prepared nanoparticles suspensions, previously filtered on a 0.45 μm sterile filter, at increasing concentrations of polymer ranging from 1 to 1000 $\mu g \cdot mL^{-1}$, medium was replaced every 48 h (Table 3.5). After the scheduled incubation time, the medium was removed and the cellular viability was estimated. For drug-loaded nanoparticles cell viability was measured on day 2 for DOX-loaded nanoparticles and day 7 for TMZ-loaded nanoparticles. Cell viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, USA). In this assay the yellow [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) is bioreduced by cells into formazan that is soluble in the culture medium with a maximum absorbance at 490

Table 3.5. Conditions for cell toxicity assays.

Derivatives	Type of NP[a]	[NP][b]	Exposition time[c]	Cell lines
PAALM-1	Unloaded	1-1000	1-72	U-87-MG, T98G, MDA-MB-231, MDA-MB-468
PMLA-Et_{100} *co*PMLA-$Et_{50}H_{50}$ *co*PMLA-$Bu_{50}H_{50}$	Unloaded / loaded	2-1000	24	U-87-MG, MDA-MB-468
*co*PAALM-L_x *co*PAALM-F_x	Unloaded	5-1000	24	U-87-MG, CRL-5904, MDA-MB-468

[a] Drug-loaded or unloaded nanoparticles.
[b] Nanoparticles concentration in the cell culture media ($\mu g \cdot mL^{-1}$).
[c] Time of exposition of cells to nanoparticles (h).

nm. This reaction only takes place when mitochondrial reductase enzymes are active, and therefore the conversion and the Abs_{490} from the 96-well plates can be directly related to cells viability.[140] Viability of untreated cells was taken as 100 %.

DOX uptake was investigated by fluorescent microscopy with cells prepared and treated as described above. After 2 h of incubation with DOX-loaded nanoparticles, cells were fixed with 4 % paraformaldehyde at room temperature for 10 min, cells nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI). The fluorescence from DOX was observed and free drug was used as positive control. For all experiments the constant concentration of DOX was 3 μM.

3.9. Measurements

DLS- Dynamic light scattering for particle hydrodynamical size and ζ-potential measurements were performed with a ZetaSizer NS (Malvern Instruments, UK) with particles suspended in deionized water. Displayed values are the average of ten readings.

DSC- Differential scanning calorimetry profiles were recorded using a Perkin-Elmer Pyris-1 (Perkin-Elmer, USA) under nitrogen flow. Thermograms were obtained from 2-4 mg samples at heating and cooling rates of 10 °C min^{-1} to determine crystallinity state and 20 °C min^{-1} to determine polymers T_g. Indium and zinc were used as standards for calibration.

Fluorescence microscopy- Cell fluorescence was accounted by using an inverted fluorescence microscope (Leica, Germany) at 40X magnification, exposure time set to 25 ms and provided with an appropriate filter set.

FT-IR- Fourier transform infrared spectras were acquired in a Perkin-Elmer Frontier FT-IR Spectrometer (Perkin-Elmer, USA) provided with a universal attenuated total reflectance (ATR) sampling accessory. Each run accumulated 4 scans.

GPC- For gel permeation chromatography a Waters 515 HPLC pump equipped with a Waters 410 Differential Refractometer detector and a Waters Styragel HR 5E column (7.8 x 300 mm) (Waters, USA) was used. Solvent consisting of 0.05 M sodium trifluoroacetate in hexafluoroisopropanol at 0.5 $mL \cdot min^{-1}$ flow rate was applied. Chromatograms were calibrated against poly(methyl methacrylate) standards (Varian, USA).

HPLC- High performance liquid chromatography was carried out with a Waters 600 system consisting of Waters 996 photodiode array detector (Waters, USA) and an Inertsil ODS-3V column (5 µm, 4.6x250 mm) (GL Sciences, USA) at an elution rate of 1 $mL \cdot min^{-1}$. Mobil phase for TMZ quantification was a mixture of methanol and 0.5 % aqueous acetic acid (10:90); and for DOX, a mixture of 0.02 M sodium hydrogen phosphate and acetonitrile (60:40). Drug concentrations were calculated with a calibration curve obtained from known amounts of free drugs as standards.

NMR- ^{1}H NMR spectras were recorded on a Bruker AMX-300 (Bruker, Germany) instrument from samples immersed in D_2O or dissolved in DMSO-d_6, $CDCl_3$ or methanol-d_4 containing minor amounts of trifluoroacetic acid. Spectra where recorded at 25 °C operating at 300.1 MHz, 128 scans were acquired with 32 k data points and relaxations of 2 s. ^{13}C NMR spectras were taken from deuterated acetone solutions with 64 k data points and 5-10 x 10^3 scans with relaxation delays of 2 s.

SEM- Scanning electron microscopy images were taken with a field-emission JEOL JSM-7001F instrument (JEOL, Japan) from

platinum/palladium coated (PMLA/DOX conjugates) and uncoated samples.

TGA- Thermogravimetric analysis measurements were performed with a Perkin-Elmer TGA-6 thermobalance (Perkin-Elmer, USA) under nitrogen flow of 20 $mL \cdot min^{-1}$ from 10-15 mg samples at a heating rate of 10 °C min^{-1}.

UV-vis- Absorbance measurements were performed using a UV-visible spectrophotometer CECIL CE 2021 (CECIL, UK) with 4 nm bandwidth. Samples were dissolved in DCM, methanol or DMSO using HPLC grade solvents. Drug concentrations were calculated with a calibration curve obtained from known amounts of free drugs as standards.

4

Poly(Methyl Malate) Nanoparticles: Formation, Degradation and Encapsulation of Anticancer Drugs

Aim and Scope

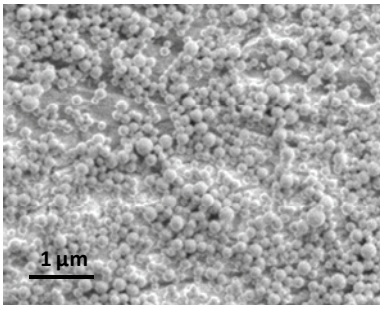
The use of poly(β,L-malic acid) for biomedical applications has shown to be advantageous for its properties as biodegradability, non-immunogenecity, and bioresorbability. Nevertheless, because PMLA water-solubility, a chemical modification of the material might be done if a solid particulate drug delivery system is desired.

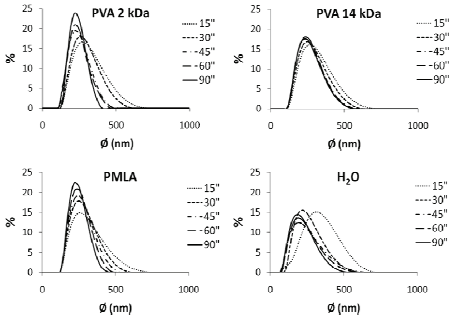
Recently, the hydrophobization of PMLA has been done by the methylation of the polymer with diazomethane. This poly(α-methyl-β,L-malate) has been used for the formulation of erythromycin-loaded microparticles by the emulsion solvent evaporation technique.

In this chapter we study the capability of poly(α-methyl-β,L-malate) for the formulation of hydrophobic nanoparticles and the encapsulation of anticancer drugs, which could have potential for crossing the blood brain barrier for brain cancer treatment.

Abstract

PMLA nanoparticles with diameters of 150–250 nm are prepared, and their hydrolytic degradation is studied under physiological conditions. Degradation occurs by hydrolysis of the side chain methyl ester followed by cleavage of the main-chain ester group with methanol and L-malic acid as the final degradation products. No alteration of the cell viability is found after 1 h of incubation, but toxicity increases significantly after 3 d, probably due to the noxious effect of the released methanol. Anticancer drugs Temozolomide and Doxorubicin are encapsulated in the NPs with 20–40 % efficiency, and their release is monitored using *in vitro* assays. Temozolomide is fully liberated within several hours, whereas Doxorubicin is steadily released from the particles over a period of 1 month.





4.1. Introduction

Biodegradable polymers and their copolymers are the preferred materials for the manufacture of a variety of devices that are nowadays widely applied in medicine and pharmacology.[19] One of their attractive potential uses is in the formulation of drug delivery systems (DDS) for parenteral administration.[5] DDS systems based on polymer particles, either nanoparticles or microparticles, are clearly advantageous because (*i*) particle size and surface can be engineered to achieve passive or active drug targeting, (*ii*) drugs can be incorporated without chemical reaction, (*iii*) drug activity is optimally preserved and (*iv*) formulation can be delivered trough different routes of administration.[80] In these systems polymer plays an essential role in the therapeutic function, because in addition to act as carrier, the system may be properly designed to control the drug delivery rate or its selective release at a specific site of action.[6]

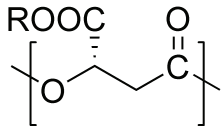
The application of DDS in chemotherapy has spread out rapidly in the last decades because their use allows to increasing drug concentration at a specific site and reducing systemic toxicity. Thus, DDS based on biopolymers or biodegradable synthetic polymers allow repeated treatment of patients without deposition and storage diseases. This has stimulated the modification of naturally occurring biopolymers and the development of new synthetic biodegradable polymers.[20,21] Synthetic poly(lactide-*co*-glycolide) (PLGA) derivatives have been the center focus of a great amount of research in the last decades due to their advantages respect to other systems.[7] Nevertheless, PLGA micro- and nanoparticles (NP) devices still retain a number of drawbacks mainly related to their releasing pattern that largely limit their applications. New options based on biopolymers able to meet specific requirements are therefore being currently searched. In this regard, poly(β-L-malic acid) (PMLA) (Scheme 4.1) and its derivatives constitute a family of promising candidates.

PMLA is a water-soluble, biodegradable, bioabsorbable, and non-immunogenic polyester[141] that can be produced either by chemical synthesis[20,142] or by fermentation of certain microorganisms.[141] The properties and functionality of PMLA are adjustable by chemical derivatization.[51] In fact, the carboxylic side group of PMLA can be modified to modulate the overall hydrophobicity of the polymer or to introduce bioactive ligands.[50] Recently, we have reported the methylation of PMLA by diazomethane[51] to create hydrophobic PMLA methyl esters suitable for the formulation of drug delivery microparticles.[48]

In cancer therapy, the treatment of tumor cells must proceed with minimal side effects to normal cells and nontoxic drug formulations should be applied.[80] The use of polymeric DDS in such therapy is increasing in popularity since they are less immunogenic than viral vectors.[50] Furthermore, DDS based on NPs offer clear advantages compared to microparticles for their potential ability to cross cell membranes. Once the polymer is selected, generation of NPs with the desired size and encapsulation efficiency are the foremost requirements to fulfill DDS design.

In this work, we wish to report the preparation of NPs from poly(α-methyl-β-L-malate) (PAALM-1), their *in vitro* degradation under physiological conditions, and their capacity for encapsulation and delivery of drugs to treat brain cancer. A major factor limiting intracranial therapeutic levels of systemically administered active agents is the restriction of permeability imposed by the blood brain barrier (BBB).[143] Temozolomide (TMZ) is widely recognized as one of the most effective antineoplastic agents for glial tumor, in great part due to its ability to cross the BBB. Some detrimental side effects have been observed however upon its prolonged systemic administration because high dosages have to be given in order to achieve the required therapeutic effect.[144] A platform based on PMLA for the design of a water soluble nanoconjugate for brain tumor treatment has been described.[59]

This nanoconjugate is known to cross the brain tumor barrier (BTB) by transcytosis using an attached antibody that binds to transferrin receptor. A similar nanobioconjugate based on PMLA for the delivery of TMZ has been recently published.[62]



Scheme 4.1. Repeating unit of PMLA (R=H) and its methyl ester PAALM-1 (R=CH_3).

4.2. Results and discussion

4.2.1. Synthesis and characterization of PAALM-1

PMLA was methylated to 100 % degree with diazomethane in dry acetone. This reaction allowed us to obtaining a complete esterification of the carboxylic side groups of PMLA without significant reduction in molecular weight. PAALM-1 precipitated from the reaction medium and was purified by repeated dissolution-precipitation in chloroform/ether. The final PAALM-1 was NMR spectroscopically pure and its weight-average molecular weight and polydispersity determined by GPC were 33 kDa and 1.4, respectively.

4.2.2. Nanoparticles formation and characterization

Spherical nanoparticles were obtained by the emulsion-evaporation method using three emulsifiers at four different concentrations. We were also able to obtain NPs without using emulsifier by fast evaporation of the organic phase under reduced pressure; under

such conditions particle could be formed before coalescence of the disperse phase occurred. The overall appearance of the obtained nanospheres is shown in the SEM pictures of Figure 4.1. Light scattering measurements indicated that they have diameters with average values ranging between 100 and 350 nm, the smallest ones being those prepared in the absence of emulsifier, and a satisfactory polydispersity.

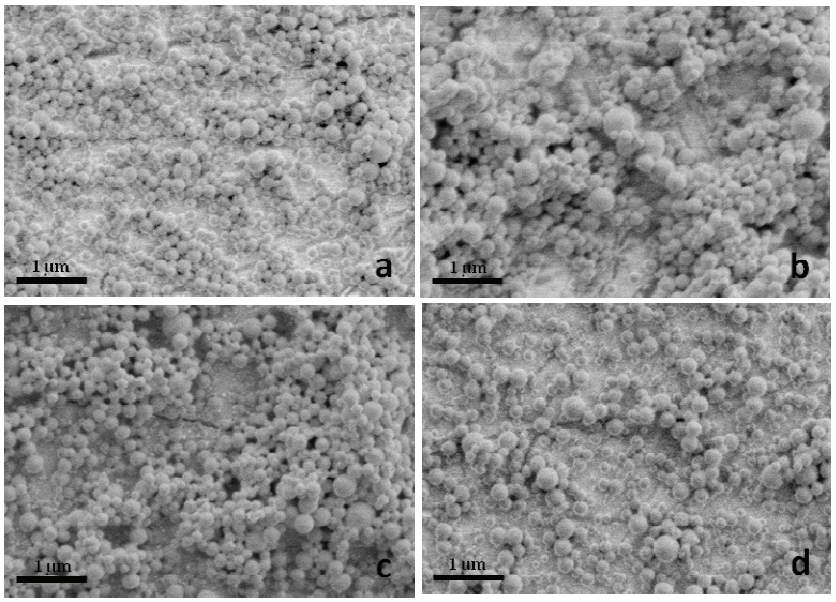


Figure 4.1. SEM micrographs of PAALM-1 nanoparticles prepared using different emulsifiers, a) PVA-2 kDa, b) PVA-14 kDa, c) PMLA and d) without emulsifier.

As it is seen in Figure 4.2, all samples show a unimodal distribution of sizes when analyzed by light scattering. The effect of emulsifier concentration on the size distribution profiles were not significant, which can be attributed to the governing effect of the solvent evaporation process. On the contrary, the influence of the applied sonication time was clearly noticeable. As demonstrated in Figure 4.2, size distribution profiles were displaced to the left and became narrower with increasing sonication time indicating that particles became smaller

and less polydisperse in size. Variation in particle size as a function of sonication time is plotted in Figure 4.3 showing that the particle diameter decreases with time approaching asymptotically to a value close to 220 nm when an emulsifier was used for preparation and around 175 nm in the absence of emulsifier. According to the previous work carried out on NPs preparation using this method,[120] such effect appears because the size of dispersed drops decreases with sonication time approaching to a minimum critical size, which is determined by the relative phase viscosities, interfacial tension between the phases and the magnitude of the emulsion generating force. In our case, where a rapid organic phase evaporation occurs and mean particle size is not affected by increasing emulsifier concentrations, it seems that it is emulsion droplet size (related to sonication time) rather than emulsion stability (related to emulsifier concentration) which determines the final particle size. This is fully consistent with the fact that NPs could be obtained in the absence of emulsifier and also with the different effect that emulsifier concentration and sonication times exerted on particle size. Particles sonicated for 45 s were chosen for the subsequent assays since their size differences are minimum. The characteristics of these NPs are compared in Table 4.1.

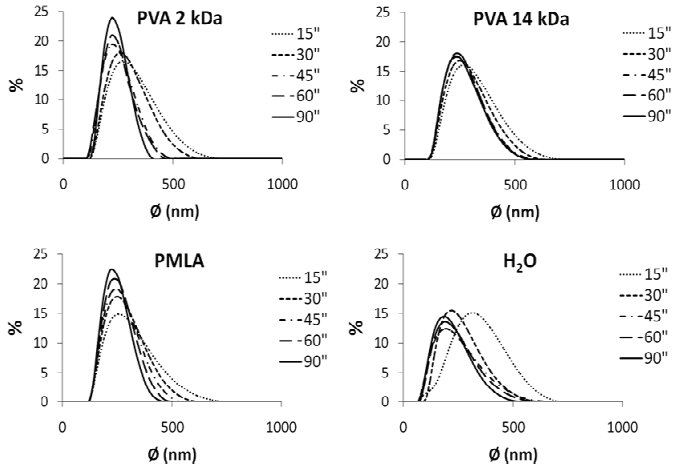


Figure 4.2. Particle size distribution as a function of sonication time for particles prepared with and without emulsifier, as indicated.

The ζ-potentials of the particles have average values between –20 and – 34 mV with the highest negative value observed for NPs that were prepared without using emulsifier. As the ζ-potential is related to surface charges, it directly affects particle's suspension stability. Usually a ζ-potential higher than 25 mV (positive or negative) is taken as the minimum to maintain the system in a stable disperse state. Our results indicate that NPs prepared using PVA as emulsifier do not reach such value whereas those prepared without emulsifier will be able to form well stable dispersions.

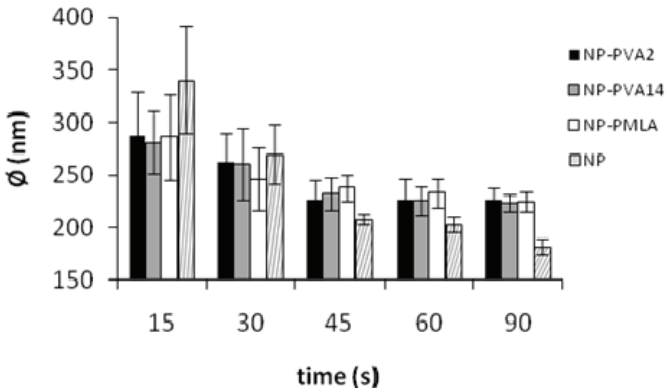


Figure 4.3. Decrease of mean particle diameter with sonication time. Error bars stand for standard deviations.

Table 4.1. Mean diameter, size polydispersity index (Pd.I.) and ζ-potential of nanoparticles used for degradation.

Nanoparticle	Emulsifier	Diameter (nm)	Pd.I.	ζ-potential (mV)
NP-PVA2	PVA 2kD	222	0.033	-23.3
NP-PVA14	PVA 14kD	231	0.193	-20.6
NP-PMLA	PMLA	238	0.063	-25.7
NP	-	207	0.236	-33.9

4.2.3. Hydrolytic Degradation

The hydrolytic degradation rate of PAALM-1, both in powder form and as NPs, was comparatively estimated by following the evolution of the molecular weight with incubation time (Figure 4.4). PAALM-1 NPs showed a degradation rate lower than the powder. After 18 weeks of incubation, when the powder appeared completely degraded, the polymer in the NPs still retained between 25 and 75 % of its original molecular weight depending on the procedure applied for emulsification. A problem associated to the use of emulsifier is its binding on the particles surface. Since emulsifier stays at the oil/water interface during solvent evaporation, it presumably remains attached to the surface of the particle altering thereby the surface composition and consequently the degradation rate. The occurrence of irreversible binding of PVA on particle surface of PLGA at the water/DCM interphase has been reported by several authors,[127,145] and the slow degradation rate observed for such particles was related to the relatively low digestibility of the PVA coating.[146] As it is shown in Figure 4.4, NPs prepared either without emulsifier or with PMLA emulsifier, degraded much faster than those prepared using PVA, which strongly supports that also in our case, PVA attached to NPs surface acts as a hydrolysis protecting coat. Nevertheless, what it is really worthy to note is that PAALM-1 NPs degrade considerably faster than those made of poly(α-benzyl β-malate), which has been reported to undergo only 40 % of molecular weight reduction after 20 weeks of incubation.[5]

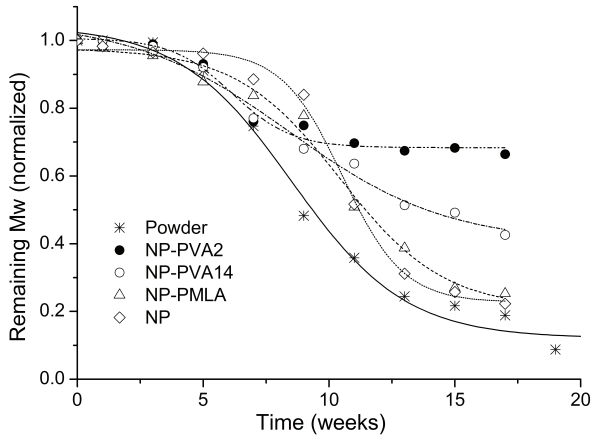
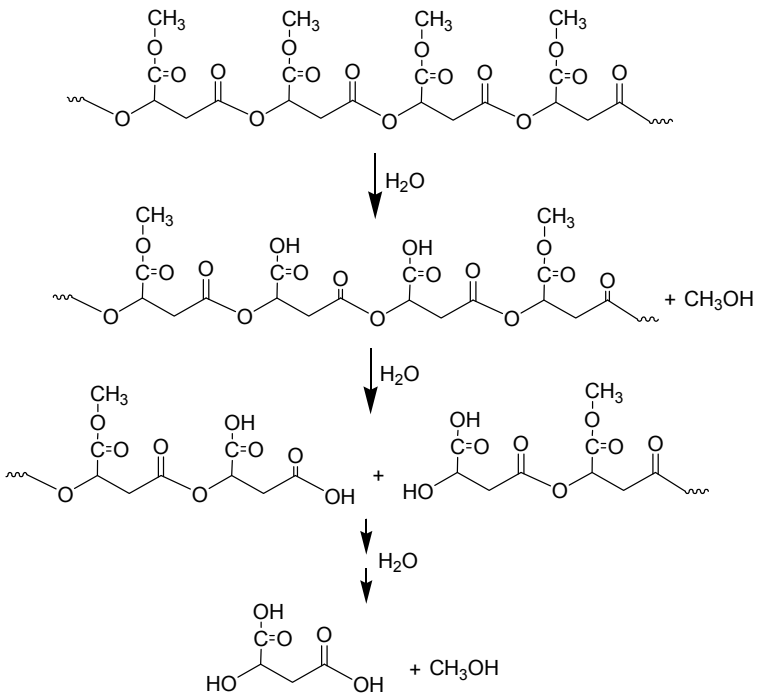


Figure 4.4. Hydrolytic degradation of PAALM-1 powder and nanoparticles in PBS pH 7.4 at 37 °C. Nanoparticles were prepared using three different emulsifiers or without emulsifier.

Hydrolytic degradation mechanism of PAALM-1 was studied in deuterated water at 60 °C by NMR analysis of the incubating medium using powder samples (Figure 4.5a). At the physiological temperature of 37 °C, hydrolysis rate was so low that signals arising from degraded products were almost undetectable in the first months of incubation. Conversely, a singlet signal corresponding to methanol, which is released in the hydrolysis of the ester side group, started to be observed after only 1 week of incubation at 60 °C. This signal increased continuously with time until complete hydrolysis of the methoxycarbonyl group. Conversely, the signal corresponding to the methyl group attached to the polymer chain appeared after 3 weeks indicating the presence of soluble chain fragments in the supernatant. This solubilized material may be oligomers or partially side chain hydrolyzed PAALM-1 long fragments. As the polymer began to be water soluble, the hydrolysis rate increased so the spectra recorded after 5 weeks of incubation showed signals corresponding to a mixture of degradation compounds including methanol, malic acid, and more or less methylated oligomers.

A similar NMR analysis carried out with PAALM-1 NPs revealed that the degradation mechanism was essentially the same as observed with powder but with noticeable differences in the timing of signal appearance. ^{1}H NMR spectra recorded at increasing incubation times from the supernatant of the NPs made with PMLA as emulsifier or without emulsifier (not shown), were very similar to the case of the powder. On the basis of the collected NMR and GPC data, and in agreement with previous results reported by us on partially methylated PMLA,[48] the basic mechanism that can be outlined for the hydrolytic degradation of PMLA is depicted in Scheme 4.2.



Scheme 4.2. Hydrolytic degradation mechanism of PAALM-1 at 37 °C.

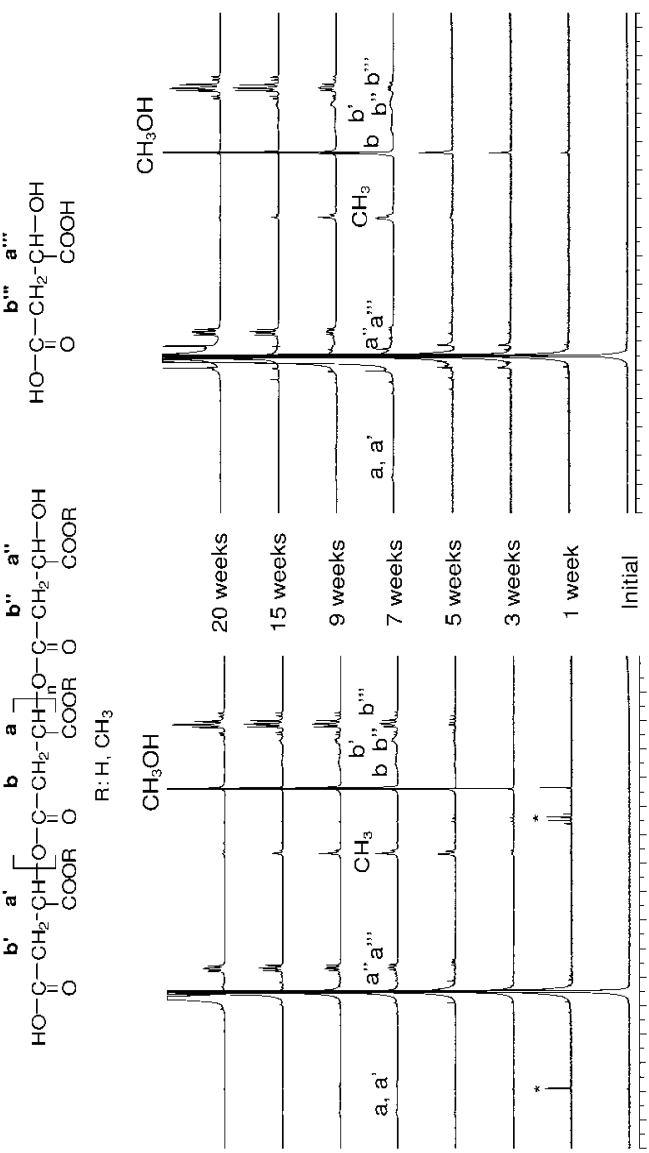


Figure 4.5. ^{1}H NMR spectra of the supernatant. Degradation in deuterated water at 60 °C of PAALM-1: a) powder, b) NP-PVA14, recorded at the indicated incubation times. *Residual solvent.

4.2.4. Cytotoxicity

For its potential use as a biomaterial it was mandatory to evaluate the toxicity of NPs made of fully methylated PMLA. Thus, an *in vitro* study of PAALM-1 NPs cytotoxicity on human glioma cell lines U-87 MG and T98G, and invasive human breast carcinoma cell lines MDA-MB-231 and MDAMB-468, was performed. Cellular viability was measured as a function of polymer concentration using the MTT test for contact times of 1, 12, 24, and 72 h between polymer and cells. As it is expected, results obtained for all cell lines indicated that the percentage of viability decreased when contact time and concentration of polymer increased (Figure 4.6). The effect of the NPs on cell viability depended also on cell line type so that glioma U-87MG and breast cancer MDA-MB-231 cells were more affected than glioma T98G cells and breast cancer MDA-MB-468 cells.

Toxicity caused by physical damage due to membrane disruption may be neglected since NPs effect on cell viability is not manifested in the time scale of 1 h. In a way similar to that observed for partial hydrolyzed PAALM-1 derivatives,[47] it is highly probable that the low toxicity observed was due to the effect of methanol that is released during polymer degradation. Both methanol and L-malic acid are generated in the hydrolysis of PAALM-1 but whereas L-malic acid is converted into water and carbon dioxide in the tricarboxylic acid cycle, methanol is known to adversely affect the living cells. Similar results have been reported for NPs made of other PMLA derivatives, where cytotoxicity was also related to the degradation products generated in the cell culture media.[21] Nevertheless, the toxicity observed for exposure times over 12 h may be considered negligible, because in DDS applications residence times for NPs in the human body will be only a few hours before they are cleared from blood through the renal system.

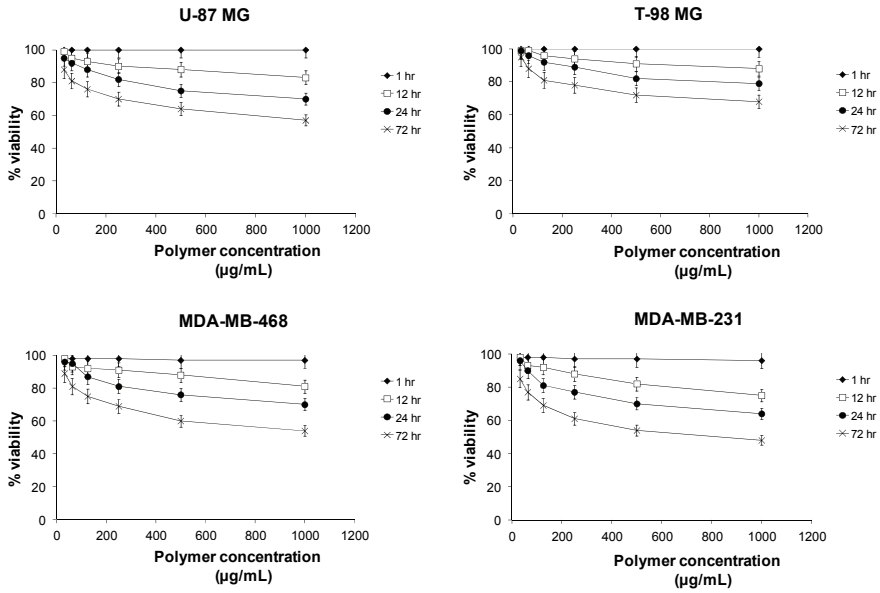


Figure 4.6. Cell viability, of human glioma cell lines U-87 MG and T98G and invasive breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 after different contact times between polymer and cells as a function of polymer concentration.

4.2.5. Drug Encapsulation and in vitro Release

TMZ and DOX encapsulation was made in PAALM-1 without using emulsifiers in order to avoid exhaustive washing and minimize drug losses. Although both drugs were encapsulated by the same method, DOX was encapsulated with a higher efficiency than TMZ (Table 4.2), due to the higher solubility of TMZ in water or the poor solvent compatibility between the drug and the polymer. Both drugs display a remarkable affinity for polar solvents which causes a diffusion of these compounds from the organic phase to the aqueous phase during emulsion's generation. In the case of DOX this phenomena could be reduced by the addition of TEA to modify DOX solubility and increase its encapsulation efficiency.

The releasing profiles of TMZ from PAALM-1 NPs obtained at different pH are shown in Figure 4.7. The analysis of TMZ release under physiological conditions is complex because above pH = 7.0 it undergoes fast hydrolysis yielding AIC together with the methyldiazonium ion, which is the chemotherapeutically active molecule.[147,148] For a correct evaluation of the *in vitro* TMZ release, it will be therefore necessary to quantify the delivery of both compounds. A releasing assay carried out at pH 6.8 revealed that a significant decomposition took place even at this pH. Nevertheless, there is meaningful differences in the AIC release profiles generated at pH 7.4 and 6.8, which clearly indicate that the formation of AIC follows the release and decomposition of TMZ from the particles. The release of TMZ seems to be independent of pH, but its half-life time appeared to be longer at pH 6.8, as it was expected. Thus, firstly TMZ was delivered from PAALM-1 NPs and then it decomposed forming AIC with the consequent release of the methyldiazonium ion.

Table 4.2. PAALM-1 nanoparticles encapsulation of TMZ and DOX.

	Drug content %[a]	**EE %[b]**
TMZ	2.18 ± 0.17	21.75 ± 1.7
DOX	4.20 ± 0.62	42.06 ± 6.2

[a] Percentage of drug contained in the nanoparticles upon encapsulation (w/w).
[b] Percentage of the drug that is encapsulated.

Under physiological conditions the release of DOX followed a much lower rate than TMZ. Whereas only a few hours were required for a complete release of TMZ the complete liberation of DOX needed more than 1 month (Figure 4.8). Since the releasing profile of DOX follows more or less closely the degradation profile of PAALM-1, it can be reasonably concluded that the delivery of this drug is governed by the hydrolysis of the polymer. On the contrary, the fast release of TMZ must happen by diffusion during the first stages of the NPs degradation. The different chemical nature of TMZ and DOX account for the remarkable differences they display in their releasing from PAALM-1 NPs.

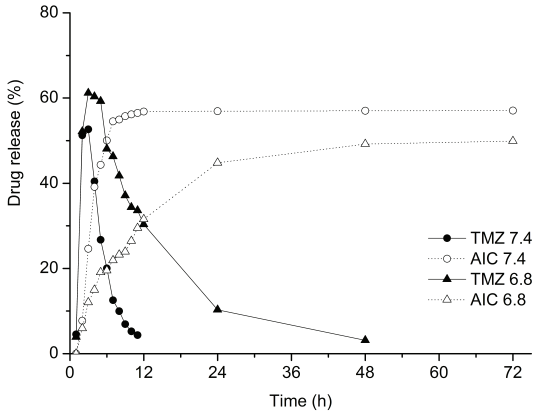


Figure 4.7. Profiles of TMZ *in vitro* release from PAALM-1 nanoparticles and formation of AIC from released TMZ at pH 7.4 and pH 6.8.

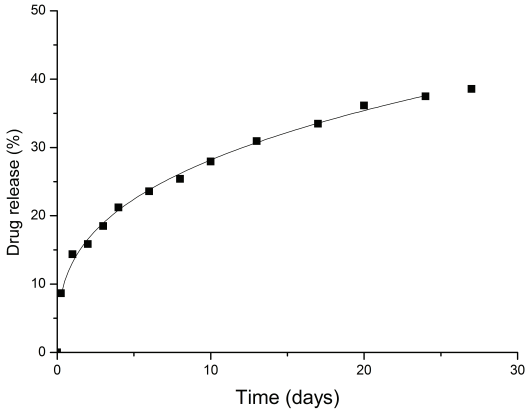


Figure 4.8. DOX *in vitro* release from PAALM-1 nanoparticles at pH 7.4.

4.3. Conclusions

Fully methylated polymalic acid obtained by methylation of fungal PMLA is a biodegradable polyester that can be used to produce nanospheres with an average diameter of around 200 nm and ζ-potential of –20 to –35 mV depending of the kind of emulsifier used in the preparation. PAALM-1 NPs prepared using polyvinylalcohol as emulsifier degraded slower than without emulsifier indicating that PVA acts as a hydrolysis protecting coat. In aqueous buffer particles hydrolyze releasing methanol followed by main chain ester bond cleavage. DOX and TMZ can be encapsulated in the NPs and released upon incubation under physiological conditions. TMZ was released within a few hours with subsequent hydrolytic pH-dependent activation resulting in AIC, while DOX was released in a time scale of days. These differences agree with the different chemical nature of both drugs. The particles described (in the absence of loaded TMZ or DOX) did not show a sign of toxicity after a few hours of administration but cell viability is significantly altered after days of contact. Although PAALM-1 NPs are suitable for either short or long time DDSs depending on the chemical nature of the drug, long residence times are expected to result in undesired side effects.

5

Nanoparticles of Esterified Polymalic Acid for Controlled Anticancer Drug Release

Aim and Scope

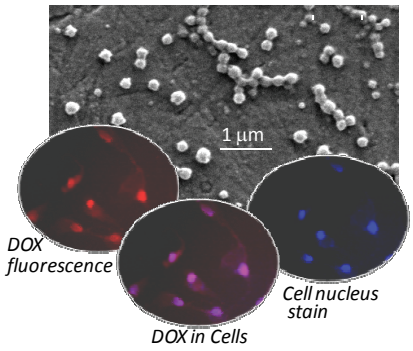
Cancer treatment with nanovehicles has increased since the elucidation of the enhanced permeation effect by cancerous tumors. This phenomenon allows the passive accumulation of nanosystems without a targeting receptor for a specific spatial delivery.

Nanoparticle formation and anticancer drugs encapsulation has been achieved with poly(α-methyl-β,L-malate), but a certain degree of cytotoxicity to prolonged exposure times was related to the methanol released during the hydrolytic degradation of the material.

The search of less cytotoxic PMLA derivatives impels this study, where PMLA is modified by esterification with short alkyl chains. The evaluation, of the synthesized derivatives, as nanoparticulate drug delivery systems of anticancer drugs is carried out.

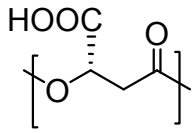
Abstract

Esterification of microbial polymalic acid was performed with either ethanol or 1-butanol to obtain polymalates conjugates capable to form nanoparticles for drug encapsulation and release. Degradation of these nanoconjugates upon incubation under physiological conditions took place by cleavage of the ester groups of both main and side chains with release of malic acid and the corresponding alcohol as unique degradation products. Fully and partially esterified polymers were used to obtain nanoparticles in the range of 100-350 nm by precipitation dialysis and emulsion solvent evaporation techniques. The anticancer drugs Temozolomide and Doxorubicin were encapsulated in nanoparticles with efficiency between 17 and 37 %, respectively. In vitro drug release essays showed that Temozolomide was almost completely discharged in a few hours while Doxorubicin was steadily released along several days. Cell cytotoxicity and cellular uptake of nanoparticles was assessed with MDA-MB468 and U87-MG cell lines. Unloaded nanoparticles did not display cytotoxicity while drug-loaded ones showed remarkable effectiveness against cancer cells. Nanoparticles made of partially ethylated polymalic acid were those that showed the highest cellular uptake.



5.1. Introduction

Nowadays, biodegradation and bioassimilation are indispensable qualities of any polymer intended for temporal applications in human therapy.[18] Accordingly biodegradable and safe polymers are the preferred materials for the manufacture of many devices that are today used in medicine and pharmacology.[19] Such requirements have stimulated efforts towards both the modification of naturally occurring biopolymers and the synthesis of new polymers with biodegradable and biocompatible properties.[20,21] In this regard, poly(β,L-malic acid) (PMLA) and its derivatives constitute a family of promising candidates. PMLA is a poly(β-hydroxy propionate) derivative with a carboxylic group stereoregularly attached to the α-carbon of the repeating unit (Scheme 5.1). The polymer is water-soluble, nontoxic, biodegradable, bioresorbable and non-immunogenic.[141,149] PMLA can be produced by either chemical synthesis[20,142] or by biosynthesis.[24] Degradation of PMLA produces easily metabolizable L-malic acid.[26] At difference from most common biodegradable polyesters such as polylactides, polyalkanoates or polycaprolactone, PMLA is a functional polymer whose properties are adjustable trough chemical modification of the pendant carboxylic side group;[51] not only the overall hydrophobicity of the polymer may be controlled by partial esterification but also bioactive ligands may be incorporated by reaction with appropriate agents.[50]



Scheme 5.1. Chemical formula of poly(β,L-malic) acid.

The application of drug delivery systems (DDS) in chemotherapy has spread out rapidly in these last years since their use reduces systemic toxicity and allows to increase drug concentration at the specific site. Although cancer cells are more vulnerable than normal cells to the effect of chemotherapy agents, drugs are nonselective and can affect normal tissues. The use of polymeric DDS in cancer therapy is increasing in popularity because they are less immunogenic than protein-based vectors, and allow repetitive administration without acute or chronic host immune response.[62] Furthermore DDS systems based on polymer particles, either nanoparticles or microparticles, are clearly advantageous by several reasons: a) particle size and surface can be engineered for passive or active drug targeting, b) drugs can be incorporated without chemical reaction, c) drug activity is optimally preserved during transportation to the site of action, and d) different routes of administration are optional for drug delivering.[2,6,80,116,117]

PMLA and its derivatives have been used either as platform in the synthesis of nanoparticles for drug delivery[18,21,53,56,95,150] or as backbone in macromolecular conjugates bearing several functionalities to treat human brain and breast tumors in mouse models.[50,59,151,152] It was concluded from these investigations that PMLA is a very suitable polymer for building efficient drug delivery systems. Recently we reported on methylated PMLA nanoparticles and showed that the cell toxicity of this system increased significantly after long time periods of incubation due to the noxious effect of the released methanol. In this work we report on other esters of PMLA, which are expected to display less cytotoxicity. These new esters are obtained by partial or total esterification of PMLA with ethanol or 1-butanol, and the nanoparticles made from them are explored for their suitability as DDS for the anticancer drugs Temozolomide and Doxorubicin.

5.2. Experimental

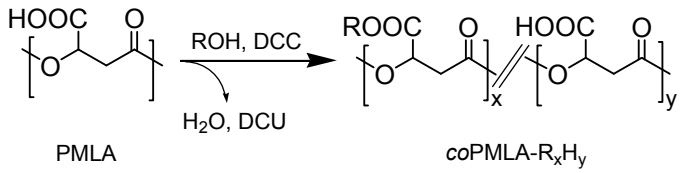
5.2.1. PMLA esterification with ethanol and 1-butanol

Esterification of PMLA was performed at room temperature with PMLA dissolved in the alcohol of choice and using dicyclohexylcarbodiimide (DCC) for activation of the carboxylic side group. Briefly, to 1 mmol of PMLA in 3 mL of either ethanol or 1-butanol, 0.5 or 1.0 mmol of DCC dissolved in 2 mL of the same alcohol, according to the desired esterification degree, were added dropwise under stirring and the reaction was left to proceed for 2 h. Exhaustive removal of dicyclohexylurea (DCU) was achieved by successive dialysis of the reaction solution against methanol for 24 h and water for 6 h using a cellulose membrane of 8 kDa cutoff. The resulting polyesters were lyophilized for recovery and storage and their conversion degree and purity ascertained by ^{1}H NMR.

5.3. Results and discussion

5.3.1 Synthesis and characterization

Ethyl and butyl PMLA esters with esterification degrees of approximately 50 % and fully esterified were obtained by reaction of PMLA with ethanol and 1-butanol, respectively, using dicyclohexylcarbodiimide as activator (Scheme 5.2). Esterification results are summarized in Table 5.1.



Derivative	R	*x*/*y*
*co*PMLA-$Et_{50}H_{50}$	Et	50/50
PMLA-Et_{100}	Et	100/0
*co*PMLA-$Bu_{50}H_{50}$	Bu	50/50
PMLA-Bu_{100}	Bu	100/0

Scheme 5.2. Esterification reaction of PMLA.

The esterification degree was controlled by adjusting the added amount of DCC so that conversions close to the used DCC/PMLA molar ratios were obtained in both cases. Reaction yields were around 50-70 % with higher values afforded in the esterification with ethanol. Product losses during polymer isolation and purification are the most probable reasons accounting for such relatively low yields. Molecular weights of esterified products were found to be higher than that of PMLA and they show a logical increasing correlation with the values that should be expected from ethyl and butyl grafting for the attained conversions. However, the experimental values are slightly lower than the theoretical ones when individually compared, and polydispersity increased noticeably. Such results seem to indicate therefore that some degradation must take place during esterification, an event that apparently was more significant when the butyl group was introduced. Nevertheless, the esterified polymers were spectroscopically pure giving ^{1}H NMR spectra in full agreement with the expected constitution and without showing

Table 5.1. Results for PMLA esterification.

	Conversion (%)	Yield (%)	M_w[a] (g·mol^{-1})	D[a]	T_g (°C)	Solubility		
						H_2O	DMSO	$CHCl_3$
PMLA	-	-	30,000		110	+	+	-
*co*PMLA-$Et_{50}H_{50}$	56	62	33,000 (33,600)	2.4	31	-	+	-
PMLA-Et_{100}	100	72	36,000 (37,200)	1.8	19	-	+	+
*co*PMLA-$Bu_{50}H_{50}$	53	57	34,000 (37,200)	2.6	13	-	+	-
PMLA-Bu_{100}	100	53	41,000 (44,500)	2.3	-12	-	+	+

[a] Weight-average molecular weights and dispersities determined by GPC. In parenthesis values estimated on the basis of the esterification degree attained.

any sign of chain end groups (see appendix 1, Fig. A1.1-A1.4); apparently low molecular weight species eventually generated by degradation were removed along the treatments applied for isolation and purification with the subsequent lowering of yields, as observed.

The reason of determining glass transition temperatures will be explained forward, but we can observe that the insertion of ethyl and butyl groups have a significant effect lowering T_g, showing a bigger effect with increasing number and length of the alkyl grafted chains, so 100 % esterified PMLA presented lower T_g than their respective copolymers (Table 5.1).

5.3.2. Hydrolytic degradation

Hydrolytic degradation assays were carried under physiological conditions (pH 7.4, 37 °C), and also in a slightly acidic medium (pH 5.0, 37 °C) intended to simulate the occurring environment inside of mature lysosomes. Results obtained by GPC for the incubated PMLA-Et_{100} and PMLA-Bu_{100} samples are compared in Figure 5.1. These polyesters after degradation produced monomodal GPC chromatograms with single peaks and values almost steadily decreasing with time following a steeper slope for the ethyl than for the butyl derivative as well as for the acidic than for the neutral solutions. Large differences were found between the two extreme cases; whereas the M_w of PMLA-Bu_{100} incubated at pH 7.4 decayed less than 10 % of the original value, a decreasing near 50 % was observed for PMLA-Et_{100} incubated at pH 5.0.

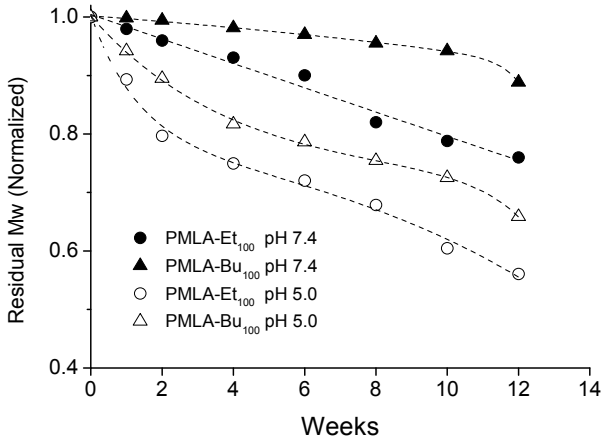


Figure 5.1. Evolution of the molecular weight of PMLA-Et_{100} and PMLA-Bu_{100} incubated in aqueous buffer at pH 7.4 and 5.0 at 37 °C.

Degradation of *co*PMLA-$Et_{50}H_{50}$ and *co*PMLA-$Bu_{50}H_{50}$ followed a more complex pattern. Bimodal or trimodal GPC chromatograms indicative of the occurrence of populations with different molecular weights were invariably registered from the residues left by these copolyesters upon incubation (Figure 5.2). To understand these results it is necessary to make clear that chains with low esterification degree are water soluble and they therefore escape the GPC analysis. Chromatograms were deconvoluted using Peakfit software to monitor the two main peak distributions with time (Figure A1.5). Taking into account the evolution of the two-deconvoluted peaks allows inferring that the one appearing at shorter retention times must represent the original polymer chains, while the second one with a much lower molecular weight must correspond to oligomeric products coming from the parent chain with a relatively high degree of esterification. The change in signal intensity of the low M_w chains between the third and fourth week of incubation suggests the occurrence firstly of significant cleavage of the initial polymer to generate oligomers and then the

degradation of these oligomers taking place at higher rate than that of the remaining higher molecular weight chains.

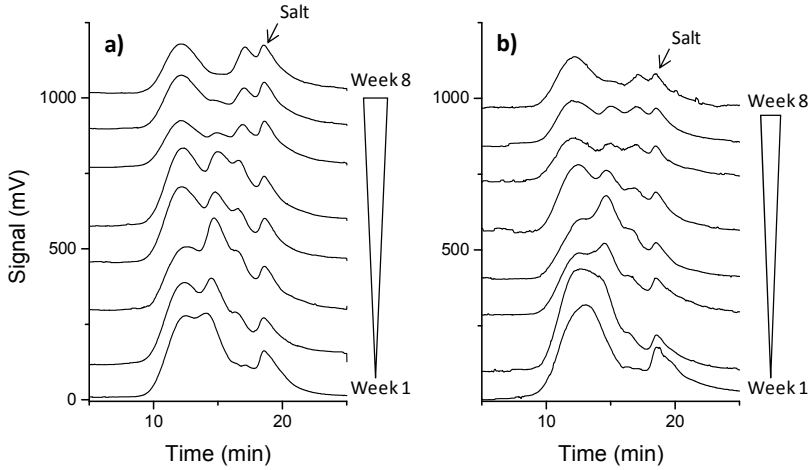


Figure 5.2. GPC chromatograms of: a) *co*PMLA-$Et_{50}H_{50}$ and b) *co*PMLA-$Bu_{50}H_{50}$, after incubation in aqueous buffer at pH 7.4 for the indicated times.

^{1}H NMR was employed for getting insight the degradation mechanism by identifying and monitoring the soluble products that are generated upon incubation of the polymers. Degradation spectra of PMLA-Et_{100} are presented in Figure 5.3. Results obtained with PMLA-Bu_{100} (Figure A1.6) were almost identical indicating that the same degradation mechanism must operate in both systems. The first detectable NMR signals appeared after three weeks of incubation and they corresponded to the alcohol (ethanol or 1-butanol) released from the hydrolysis of the lateral chain ester group. Differences between the two polymers started to be appreciated after week 8th. At this time, the spectrum recorded from the PMLA-Et_{100} degradation medium showed the signals characteristic of the ethyloxycarbonyl group while those of the butyloxycarbonyl group did not appear in the incubation medium of PMLA-Bu_{100} until week 13th. These results are a clear indication of the faster degradation and/or easier solubilization that takes place in

PMLA-Et_{100} compared to PMLA-Bu_{100}. Signals corresponding to PMLA oligomers, partially esterified polymer and free malic acid are present in both samples after week 13th but displaying higher intensity in the case of the ethyl derivative. After twenty weeks of incubation of PMLA-Et_{100}, ethanol and free malic acid were the only products detected in the incubation medium of PMLA-Et_{100}. In the degradation of PMLA-Bu_{100}, signals corresponding to the butyloxycarbonyl group were still observable at week 25th according to the higher reluctance of this group to be hydrolyzed.

Degradation of copolymers proceeded following a pattern similar to homopolymers but at higher rates since their unmodified carboxylic units confer them a marked hydrophilicity. ^{1}H NMR spectra of *co*PMLA-$Et_{50}H_{50}$ incubated at pH 7.4 and 37 ºC for three months are presented in Figure 5.4. Degradation started after only a week of incubation as it is revealed by the spectrum recorded at that time from the incubation medium. Signals indicative of the presence of ethanol, malic acid and oligomeric species are detected in the spectrum recorded from the supernatant after just a week of incubation. After two weeks, signals arising from terminal groups increased whereas those arising from the main chain diminished, which is taken as an unequivocal indication of the occurrence of main chain breaking with generation of oligomers. These oligomers still contain unhydrolysed malate units as it is revealed by the presence of signals arising from ethyl and butyl side groups. At the third week signals from free alcohol and malic acid increased in intensity, while those from the alkyl esters and oligomers became weaker as it is expected to result from the progressive hydrolysis of the lateral and main chain ester bonds. After week 6th, the only observable signals are those arising from malic acid and the alcohol, which are obviously the final products of degradation. Note that the weakening observed of signals for the alcohols over time is due to partial evaporation of these volatile compounds. A similar mechanism was concluded that must be operating in the degradation of *co*PMLA-$Bu_{50}H_{50}$ (Figure A1.7).

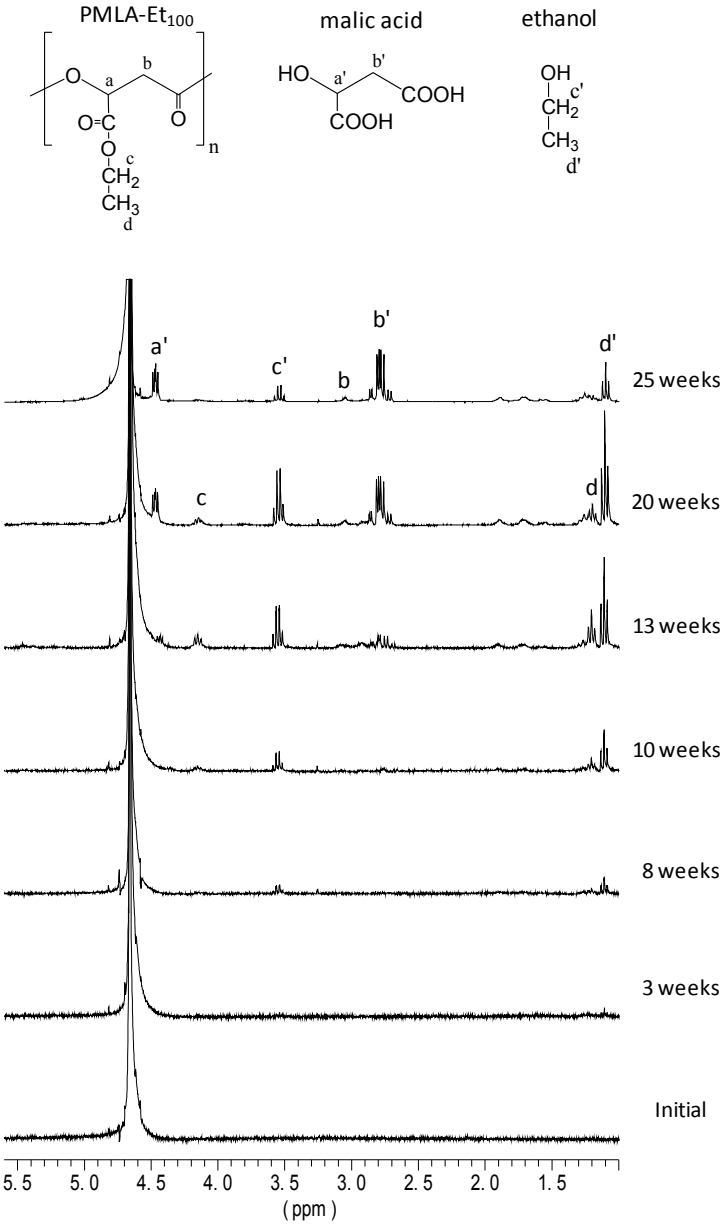


Figure 5.3. ^{1}H NMR spectra of the degradation media over time for PMLA-Et_{100} at pH 7.4.

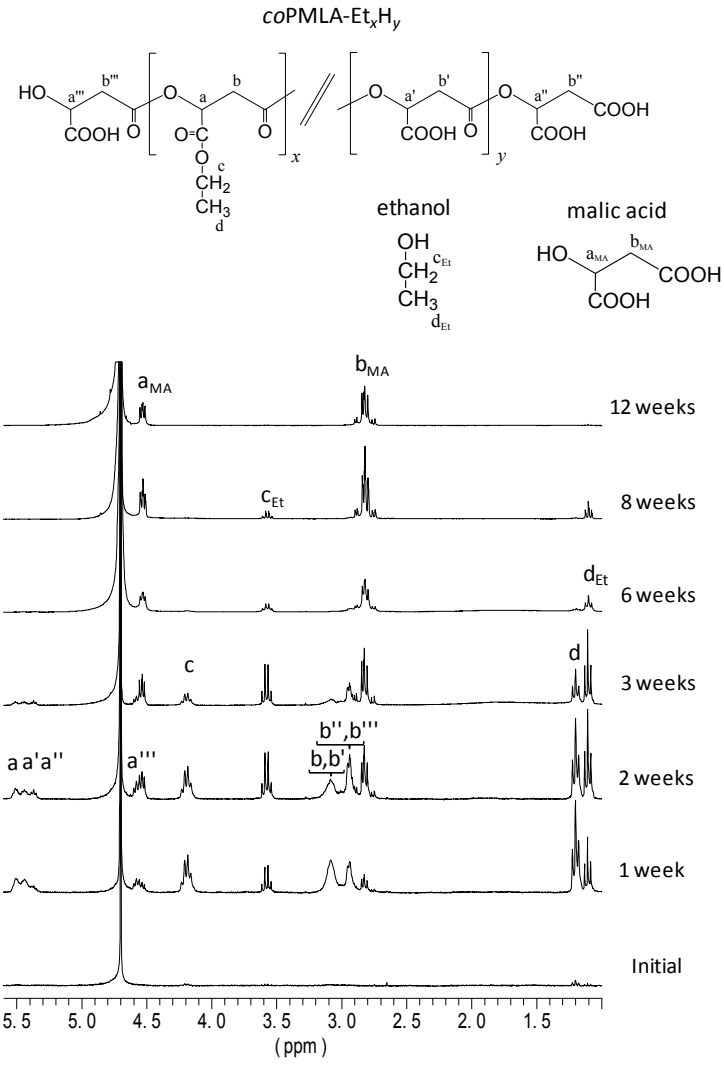


Figure 5.4. ^{1}H NMR spectra of the degradation media over time for *co*PMLA-Et$_{50}$H$_{50}$ at pH 7.4.

5.3.3. Nanoparticle formation

Given the potential application intended for these polyesters as DDS, their capability to form nanoparticles was assessed. Due to differences in polarity between fully and partially esterified PMLAs, two

different methods were employed for nanoparticle formation. The emulsion solvent-evaporation method was preferred for the most hydrophobic homopolymers (PMLA-Et_{100} and PMLA-Bu_{100}), whereas for the copolymers *co*PMLA-$Et_{50}H_{50}$ and *co*PMLA-$Bu_{50}H_{50}$, which are amphiphilic polymers, the precipitation dialysis method was instead employed. According to what is largely experienced in the self-assembly of polymers, formation of nanoparticles composed of an inner hydrophobic core and an outer shell made of hydrophilic groups should be expected.[84]

Dynamic light scattering (DLS) measurements (size distribution profiles in Figure A1.8) revealed that particles of nanometric size with average hydrodynamic diameters in the 250-350 nm range were obtained for the homopolymers by using the emulsion-evaporation method (Table 5.2). Much smaller particles with average diameters between 100 and 200 nm were obtained from the copolymers by precipitation dialysis method. Moreover, SEM observations revealed significant morphological differences among them. As shown in Figure 5.5, nanoparticles with pretty defined spherical shape were observed for all the polymers except for PMLA-Bu_{100} (Figure 5.5b). In this case the particles displayed a much larger size than that determined by DLS and their shapes were not well outlined. This is interpreted as the result of the coalescence probably occurring upon deposition of the particles on the support used for sample preparation. Such particular behavior displayed by PMLA-Bu_{100} is in agreement with its relative low T_g (Table 5.1) and its homogeneous constitution. The combination of these two factors could render sticky particles prone to coalesce when in contact with each other. Some signs of a similar behavior are also detected for PMLA-Et_{100} in Figure 5.5a although in this case the particles still retain their spherical shape. Given the unsatisfactory behavior of PMLA-Bu_{100} particles, they were discarded in subsequent drug encapsulation and release assays.

Table 5.2. Particle size and polydispersity of nanospheres formed with the different polymers.

	Average diameter (nm)	Polydispersity	Preparation method	TMZ		DOX	
				Cont.[a] %	EE[b] %	Cont.[a] %	EE[b] %
PMLA-Et_{100}	279 ± 27	0.114 ± 0.044	Emulsion-evaporation	3.75 ± 0.14	37.5	3.70 ± 0.21	37.0
PMLA-Bu_{100}	345 ± 33	0.059 ± 0.010	Emulsion-evaporation	-	-	-	-
*co*PMLA-$Et_{50}H_{50}$	136 ± 25	0.221 ± 0.038	Precipitation-dialysis	1.36 ± 0.09	13.6	1.71 ± 0.07	17.1
*co*PMLA-$Bu_{50}H_{50}$	163 ± 28	0.116 ± 0.084	Precipitation-dialysis	2.03 ± 0.02	20.3	3.27 ± 0.13	32.7

[a] Nanoparticles drug content (% w/w).
[b] Encapsulation efficiency.

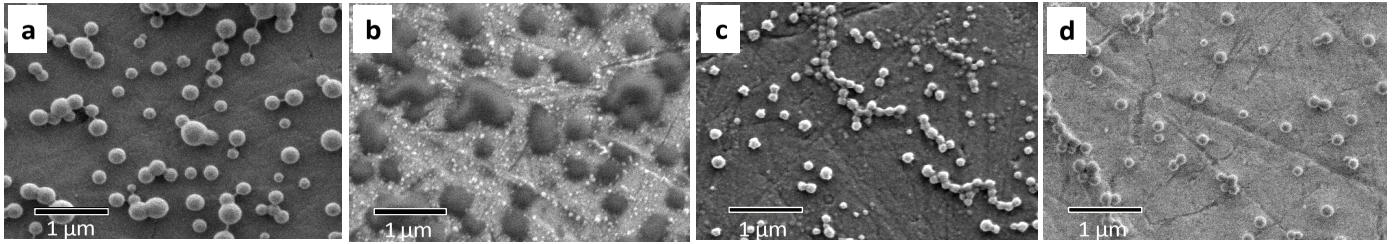


Figure 5.5. SEM micrographs of polymer nanoparticles: a) PMLA-Et_{100}; b) PMLA-Bu_{100}; c) *co*PMLA-$Et_{50}H_{50}$; d) *co*PMLA-$Bu_{50}H_{50}$.

5.3.4. Drug encapsulation and in vitro release

For encapsulation of Temozolomide and Doxorubicin in the nanoparticles, the same procedure as for nanoparticle formation was applied but with the drug added to the initial polymer solution. Results obtained for esterified PMLA excluding PMLA-Bu_{100}, are presented in Table 5.2 for the two drugs. Encapsulation efficiencies (EE) in the 13-38 % and 17-37 % ranges were achieved for TMZ and DOX, respectively. It is remarkable that EE values are very similar for both drugs encapsulated in PMLA-Et_{100}, and that higher values were attained when the emulsion method was used. Low encapsulation efficiencies and drug contents obtained for copolymers could be explained by drug loses taking place during solvent removal by dialysis for nanoparticle formation. The higher content obtained for DOX compared to TMZ in the partially esterified PMLA particles is probably due to the capability of this cationic drug to form ionic complexes with the carboxylic groups remaining present in the copolymer, in a similar manner as it has been reported to occur in other polyelectrolyte systems.[103] Limited drug loading and burst drug release are features usually associated to encapsulation and delivery in nanoparticles due to their small available volume and large surface area.[116]

Drug release was measured under close to physiological conditions, *i.e.* at pH 7.4 and 37 °C. It should be taken into account that TMZ is susceptible of degradation in water at pH 7 with generation of AIC; the half-life time of TMZ under such conditions is 2 h.[148] Both compounds must be monitored therefore in order to evaluate the actual release of TMZ. In Figure 5.6 it is shown that the maximum TMZ release took place between 2 and 4 h of incubation with a cumulative release of 60 % of the content in the case of *co*PMLA-$Et_{50}H_{50}$. Later the TMZ concentration peak decreased drastically due to its decomposition whereas the AIC concentration increased to reach a constant level after 24 h. It is worthy to note that polymer nanoparticles still released TMZ after 24 h of incubation. Comparison of the releasing profiles for the

three polymers revealed that most of the drug was delivered within the first few hours of incubation, and that the release was faster as the hydrophilicity of the polymer increased. In fact, traces of TMZ releasing from PMLA-Et_{100} nanoparticles were detected even after 48 h of incubation (Figure 5.6). Such high retention and resistance against hydrolysis of TMZ loaded in these particles is probably due to the higher hydrophobic character of ethyl polymalate.

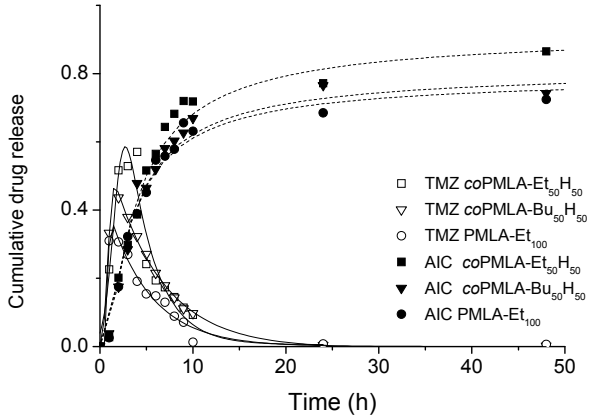


Figure 5.6. Temozolomide release from polymer nanoparticles at pH 7.4 and 37 ºC.

DOX was released much slowly that TMZ following a continuously increasing profile that after several days reaches a constant value (Figure 5.7). *co*PMLA-$Bu_{50}H_{50}$ shows the fastest release while PMLA-Et_{100} shows the slowest one. It is also remarkable that release differences among the polymers in the case of DOX are larger than for TMZ.

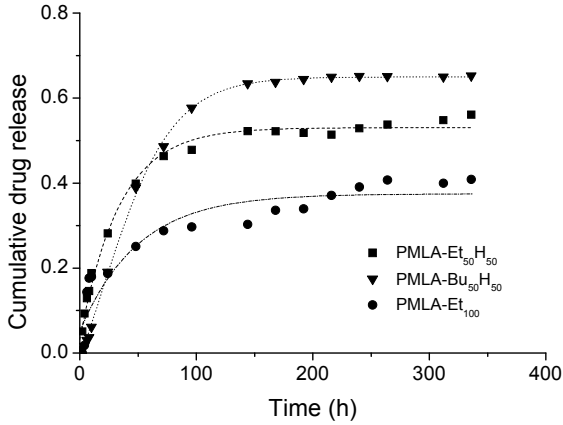


Figure 5.7. Doxorubicin release from polymer nanoparticles at pH7.4 and 37 °C.

5.3.5. Cell viability and nanoparticles cellular uptake

Cytotoxic tests were performed with unloaded and loaded drug nanoparticles on cell lines U87-MG and MDA-MB468; U87-MG cells are used as an in vitro model of human glioblastoma, while MDA-MB468 is a cell line for breast carcinoma cells. Both are extensively used to investigate the cytotoxic effect of chemotherapeutic drugs towards cancer cells. Cytotoxicities of unloaded nanoparticles were practically negligible, since at the tested concentrations cell viability remained above 94 %, except for PMLA-$H_{50}Bu_{50}$ which caused decays to 90 % viability for MDA-MB468 cell line at higher concentrations (Figure 5.8). The cytotoxicity of these derivatives (ethyl and butyl polymalates) results to be significantly lower than that observed for methyl polymalate which cell viability decayed to less than 80 % after 24 hour of exposure of the cells to the nanoparticles. The higher toxicity displayed by the methyl derivative was attributed to the action of methanol released during polymer degradation.[153]

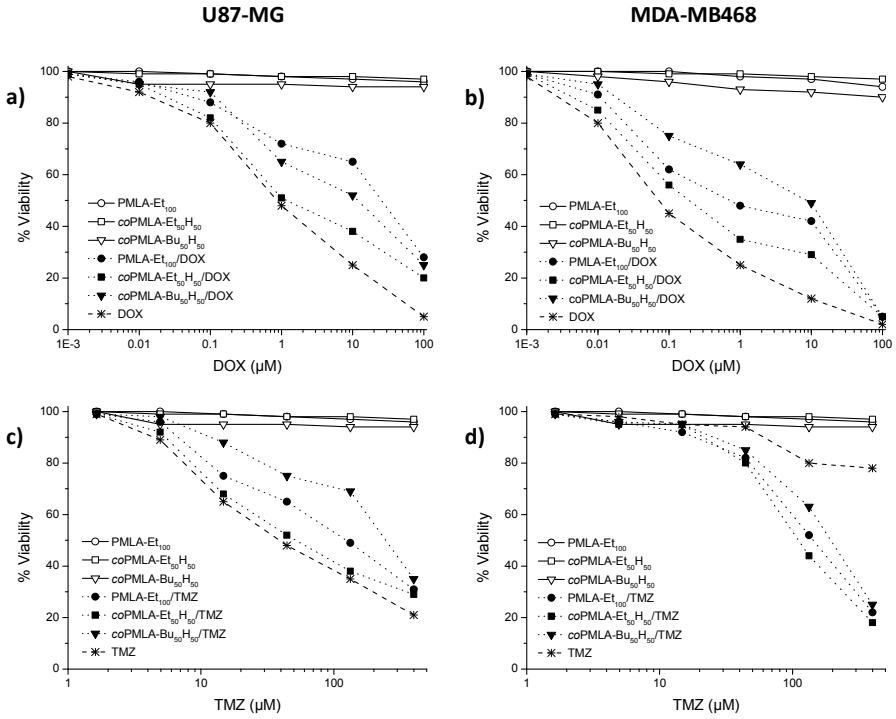


Figure 5.8. Cytotoxicity test of unloaded and drug-loaded nanoparticles and free drugs on U-87-MG and MDA-MB468 cell lines. a, b), Doxorubicin treatment and c, d), Temozolomide treatment.

In toxicology, half maximal effective concentration (EC_{50}) refers to the concentration of a drug where 50 % of its maximal effect is observed or where 50% of the population exhibits a response, after a specified exposure time, in our case 50% of viability after 2 or 7 days depending on cell line, after been exposed 24 h to the treatment. Cytotoxicity response on cell lines is plotted in Figure 5.8, as a function of drug concentration, and EC_{50} values are summarized in Table 5.3. In general loaded nanoparticles need more concentration to exert the same effect than the free drugs, with *co*PMLA-$Et_{50}H_{50}$/drug showing the closest behavior to free drugs. Second in response are PMLA-Et_{100} nanoparticles except for U87-MG cell line with DOX, in which *co*PMLA-$Bu_{50}H_{50}$/DOX presented lower concentrations for EC_{50}. The most remarkable case is

observed for MDA-MB468 cell line treated with TMZ and TMZ-NPs, in which free TMZ showed ineffectiveness at all concentrations, while TMZ loaded nanoparticles reach EC_{50} between 100-200 μM TMZ concentrations. This difference can be due to two different phenomena, first to a more extended TMZ presence in the media since free TMZ has a half life of 2 h in aqueous media, and second, to NP internalization by cells and TMZ release in the cytosol making the cells more sensitive to the drug.

Table 5.3. Cytotoxicity EC_{50} values for free and encapsulated drugs in polymer nanoparticles.

	TMZ		**DOX**	
	U87-MG (μM)	**MDA MB468 (μM)**	**U87-MG (μM)**	**MDA MB468 (μM)**
Free drug	37	> 400	1	0.08
PMLA-Et_{100}/Drug	133	130	30	0.8
PMLA- $Et_{50}H_{50}$/Drug	50	105	1	0.2
PMLA- $Bu_{50}H_{50}$/Drug	260	200	10	10

To evaluate the cellular uptake and trafficking of DOX loaded nanoparticles in U87-MG cells, we performed microscopic studies based on red autofluorescence of DOX (Figure 9). DOX loaded *co*PMLA-$Et_{50}H_{50}$ nanoparticles showed the most intense auto-fluorescence compared to the other samples; this auto-fluorescence is localized in the cytoplasm and cell nucleus, while cells treated with free-DOX demonstrated significantly less auto-fluorescence and only inside the nucleus. Loaded nanoparticles from *co*PMLA-$Bu_{50}H_{50}$ and PMLA-Et_{100} showed a limited internalization what is consistent with cytotoxicity results. The effective protection of TMZ against degradation, the slow release of DOX, the low citotoxicity and the effective internalization mainly of *co*PMLA-$Et_{50}H_{50}$

nanoparticles, make this derivative a potential material for the encapsulation and delivery of drugs for cancer treatment.

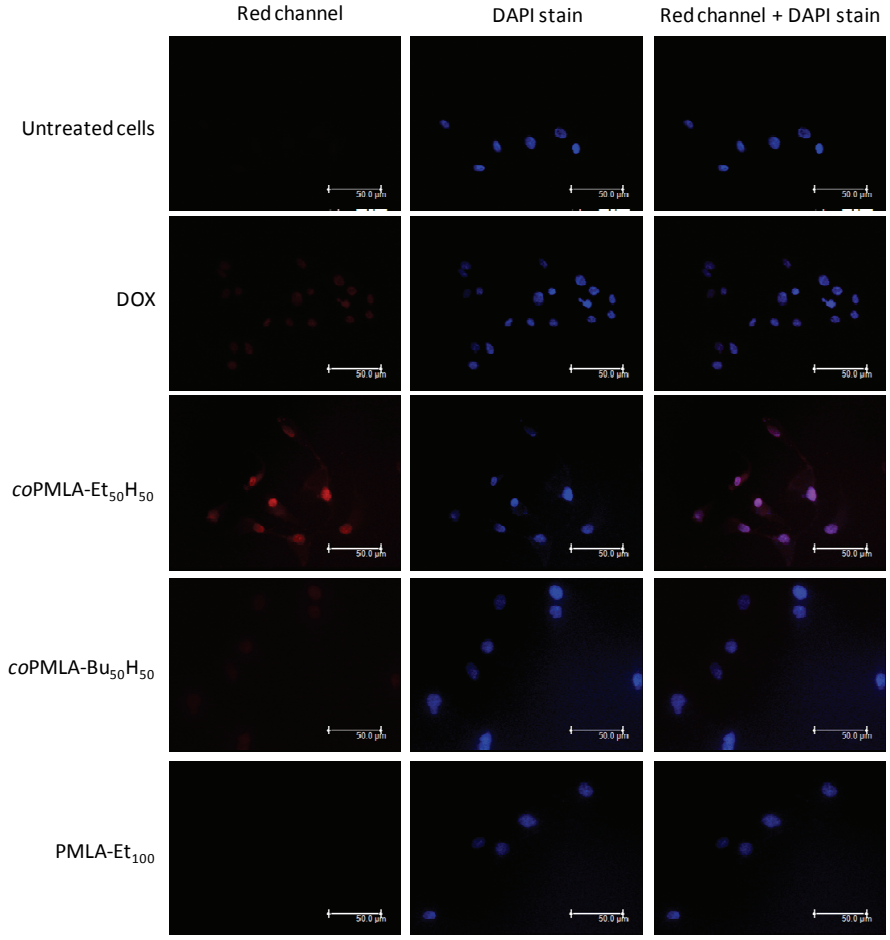


Figure 5.9. Fluorescence microscopy of U87-MG cells incubated with free DOX and DOX-loaded nanoparticles. DOX autofluorescence in the red channel.

5.4. Conclusions

Microbial polymalic acid both fully and partially esterified with ethanol or butanol are easily hydrolysable polyesters than can be employed for building nanoparticles suitable for drug encapsulation as drug delivery systems. The final degradation products of these polymers are the innocuous corresponding alcohol and easily metabolyzable malic acid. Nanoparticles diameters oscillate from 100 to 350 nm depending on the polymer and on the methodology used for particle formation and encapsulation. DOX and TMZ can be encapsulated in these nanoparticles and released upon incubation under physiological conditions. Most of the TMZ was released within a few hours with subsequent hydrolytic degradation into AIC, while DOX was steadily released in a time scale of days. Furthermore TMZ encapsulation afforded protection to the drug against hydrolytic decomposition. Drug-unloaded nanoparticles were not cytotoxic for the tested cell lines, whereas drug-loaded nanoparticles were cytotoxic for cancer cell lines. In the case of MDA MB468 cells, drug loaded particles were highly efficient, while free TMZ did not show a measurable effect. The most efficient polymer nanoparticles were *co*PMLA-$Et_{50}H_{50}$ which showed better internalization of DOX by cells than the free drug.

6

Modification of Microbial Polymalic Acid with Hydrophobic Amino Acids for Drug Releasing Nanoparticles

Aim and Scope

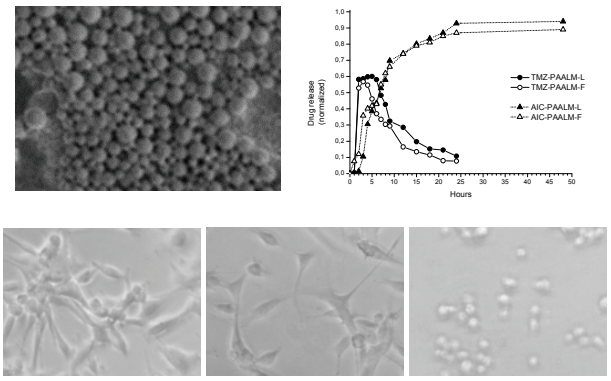
As a promising material for the design of drug delivery systems; PMLA and its derivatives have been used as platform of nanocarriers or as a constituent in macromolecular conjugates for drug delivery. The capability of PMLA to form nanoparticles and encapsulate active substances has been proved by two different methods.

Particles differ in terms of drug loading capacity, particle and drug stability, drug release rate, targeted delivery ability and toxicity. As we increase the diversity of materials we also augment the possibility and range of application of the systems.

This chapter treats about the use of bioorganic molecules, amino acids, for modification/hydrophobization of PMLA for nanoparticle generation. This kind of modification/functionalization has been used before in DDS for hydrophobization or to confer membranolytic properties to the systems.

Abstract

Microbial poly(β,L-malic acid) was modified with either L-leucine ethyl ester (L) or L-phenylalanine methyl ester (F) to produce amphiphilic copolymers. The degradation of these copolymers in aqueous buffer took place under physiological conditions in a few weeks by hydrolysis of the side chain ester group followed by cleavage of the main chain with releasing of soluble oligomers and L-malic acid. Spherical nanoparticles with diameters ranging between 70 and 230 nm were prepared from these copolymers by the dialysis-precipitation method. No alteration of the cell viability was observed after 24 h of incubation of these nanoparticles in different cell lines provided that concentrations were maintained below 0.125 % (w/v). Anticancer drugs Temozolomide and Doxorubicin were encapsulated in nanoparticles with 15-30 % efficiency. Drug release from the nanoparticles in aqueous buffer was monitored in vitro; Temozolomide was released within several hours whereas Doxorubicin took several weeks to be completely liberated.



6.1. Introduction

Contemporary cancer therapy is in urgent need of increasing the treatment efficiency. Although cancer cells are more vulnerable than normal cells to the effect of chemotherapy agents, drugs are nonselective and unavoidably affect normal tissues. Research is now focused on killing cancer cells using more specific targeting because toxicity of normal cells is the main constrain for dose and frequency, both being critical important factors in determining the efficiency of the cancer chemotherapy treatment.[119] In the last decades, drug delivery systems (DDS) based on biodegradable polymeric nanoparticles (NPs) have received great attention as effective carrying devices. Nowadays, biodegradation is considered a prerequisite for any high molecular weight material which is to be introduced in a living body for a limited period of time.[18]

Nanoparticles for drug delivery include numerous architectural designs in terms of size, shape, and materials. Particles differ in terms of drug loading capacity, particle and drug stability, drug release rate, and targeted delivery ability.[119] A variety of polymers have been tested and proved to deliver the drug to a target site thus increasing the therapeutic benefit while minimizing side effects.[6] Polymer based delivery systems are usually preferred because they are less immunogenic than protein-based ones, and they allow repetitive treatments without acute or chronic host immune response, which is a major requirement for the effective cancer treatment.[62]

Poly(β,L-malic acid) (PMLA), a biologically produced polyester, has a great potential in biomedicine because its excellent biodegradability and biocompatibility. PMLA is readily biodegraded producing easily metabolizable L-malic acid.[26] Unlike other biodegradable polymers, PMLA can be chemically modified through derivatization of the carboxylic side groups to change and modulate its properties.[95] PMLA and its derivatives have been used as platform in the

synthesis of nanocarriers for drug delivery,[21,53,56,153] or as a constituent in macromolecular conjugates bearing several functionalities to treat human brain and breast tumors in mouse models.[50,59,151] In all of these investigations it has been concluded that PMLA is a promising building block for the design of efficient drug delivery systems.[49]

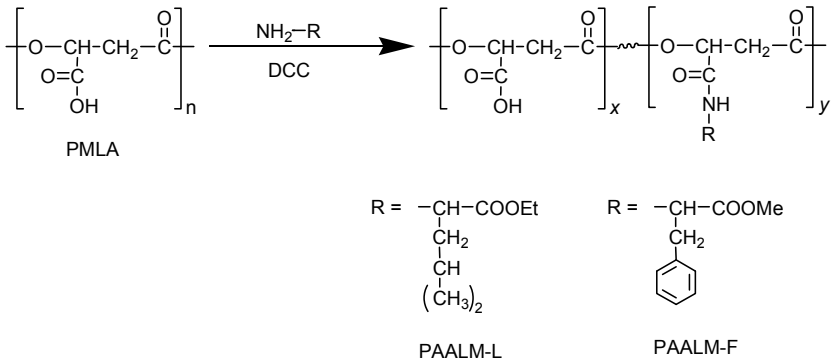
Unmodified PMLA is highly hydrophilic and readily soluble in water due to its carboxylic polyfunctionality. Grafting of hydrophobic amino acids or peptides on hydrophilic polymers has been done to give an amphiphilic character to the polymer so it could form NPs,[84] or to introduce a membranolytic shell which helps DDS to escape from endosomes to cytoplasm.[59] In this work, with the aim of inducing amphiphilic character, PMLA has been subjected to partial amidation with alkyl esters of L-leucine (L) (PAALM-L) and L-phenylalanine (F) (PAALM-F). The partially amidated polyesters were used for preparing self-assembled nanoparticles and the suitability of these as drug delivery systems has been examined.

6.2. Experimental

6.2.1. Synthesis of poly(β,L-malic acid)-graft-AA

PMLA was conjugated with L-leucine ethyl ester or L-phenylalanine methyl ester, by activation of the carboxylic side groups with dicyclohexylcarbodiimide (DCC) (Scheme 6.1). Briefly, 1 mmol of PMLA was dissolved in 4 mL of acetone at room temperature (RT) and the solution cooled in an ice bath. The amino acid used for grafting and DCC in 1 mL of acetone were added dropwise. The amino acid and DCC amounts depended on the conversion degree that was desired (Table 6.1). Reaction was left to proceed under stirring for 1 h at 4 °C and then for 23 h at RT, after which the reaction mixture was cooled in the freezer and the precipitated dicyclohexylurea (DCU) removed by filtration. To

remove DCU traces remaining in solution, the filtrated polymer solution was dialyzed against methanol for 24 h using a cellulose membrane with a cutoff of 8 kDa. The modified PMLA was recovered from the dialyzed solution by adding water and subsequent freeze-drying.



Scheme 6.1. Amidation reaction of PMLA using DCC as activating agent.

6.3. Results and discussion

6.3.1. Amino acid grafting on PMLA

PAALM-L and PAALM-F copolymers nominally containing 30, 60 and 90 % of amidated units were obtained at yields in the 55-65 % range by reaction of PMLA with the esterified amino acids L-leucine and L-phenylalanine using DCC as carboxylic group activator (Table 6.1). Conversions attained were precisely assessed by ^{1}H NMR (Figure 6.1) and the GPC analysis showed that the initial polymer did not undergo significant reduction in the molecular weight. All copolymers are soluble in dimethylsulfoxide, acetone and HFIP but non-soluble in diethyl ether and water. Only the copolymers amidated at 90 % were soluble in chloroform.

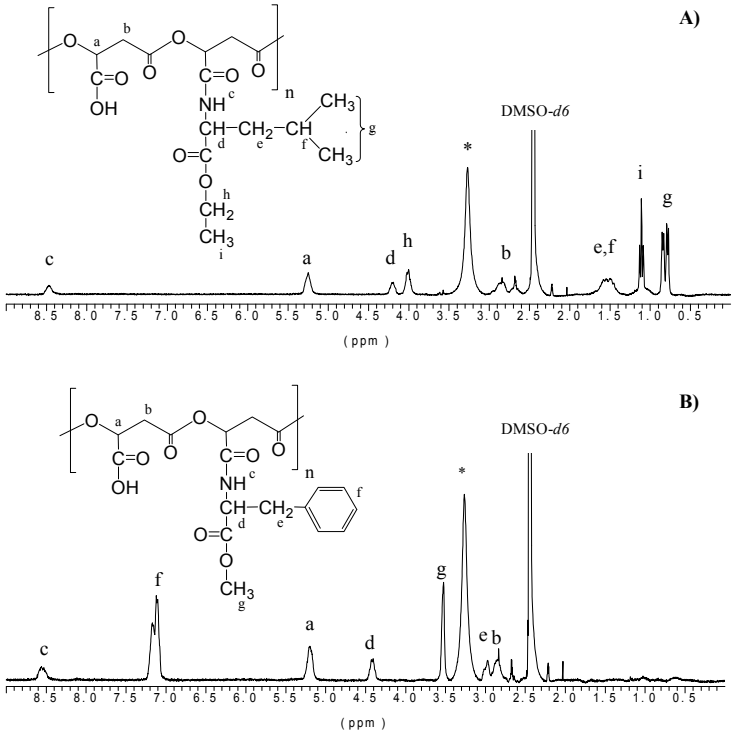


Figure 6.1. ^{1}H NMR spectra of: A) PAALM-L60 and B) PAALM-F60. (*) Peak of water.

Unfortunately, the ^{13}C NMR spectra recorded from these copolymers did not provide the information required to carry out a statistical analysis of the chain microstructure. Nevertheless, the evolution of the signal arising from different carbons of the amino acid moiety units (a, a′) with changes in composition revealed that the distribution of amidated and free-carboxylic units along the copolymer chain must be essentially at random. As it is illustrated in Figure 6.2, this signal evolves from an essentially single peak for the 90 % amidated PMLA to a doublet for both the 30 and 60 % amidated copolymers with relative peak intensities changing according to composition. The observed splitting is interpreted as due to the presence of dyads made of amidated-amidated (LL or FF) and amidated-non-amidated (*L*M/M*L* or M*F*/*F*M) units, which is an indication of a random microstructure.

Table 6.1. Results of the PMLA modification reaction.

Copolymer	AA[a]	PMLA:DCC:AA[b]	Reaction results		GPC	Solubility[c]						
			Conversion (%)	Yield (%)	M_w	A	B	C	D	E	F	G
PAALM-L30	L-Leu	1:0.5:1	28	57	34,400	+	+	+	+	-	-	-
PAALM-L60	L-Leu	1:1:1	58	56	33,600	+	+	+	+	-	-	-
PAALM-L90	L-Leu	1:1.5:2	88	65	33,700	+	+	+	+	+	-	-
PAALM-F30	L-Phe	1:0.5:1	27	56	32,900	+	+	+	+	-	-	-
PAALM-F60	L-Phe	1:1:1	55	63	31,800	+	+	+	+	-	-	-
PAALM-F90	L-Phe	1:1.5:2	89	60	34,500	+	-	+	+	+	-	-
PMLA		-	-	-	30,000	+	+	+	-	-	-	+

[a]Amino acid.
[b]Reagent molar ratios used in the amidation reaction.
[c]A: DMSO, B: methanol, C: acetone, D: HFIP, E: chloroform, F: diethyl ether, G: water.

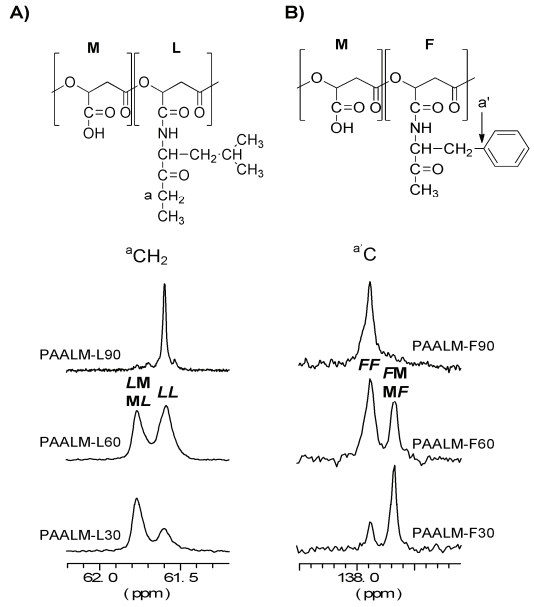


Figure 6.2. ^{13}C NMR spectra of the amino acid region corresponding to $^{a}CH_2$ of PAALM-L (A) and non protonated aromatic carbon of PAALM-F (B) of copolymers with different conversion degrees.

6.3.2. Hydrolytic degradation

The hydrolytic degradation of the copolyesters was performed under physiological conditions (pH 7.4, 37 ºC), and the process was followed by GPC of the residue and by ^{1}H NMR of the released products. According to expectations, GPC results showed that degradation rate decreased with the increasing amidation degree of the copolyester and results were similar for both Leu and Phe derivatives. As it is shown in Figure 6.3, the 30 % amidated copolyesters become fully degraded after four weeks of incubation whereas for the 90 % amidated copolymers, the reduction in molecular weight was less than 30 % after six weeks of treatment.

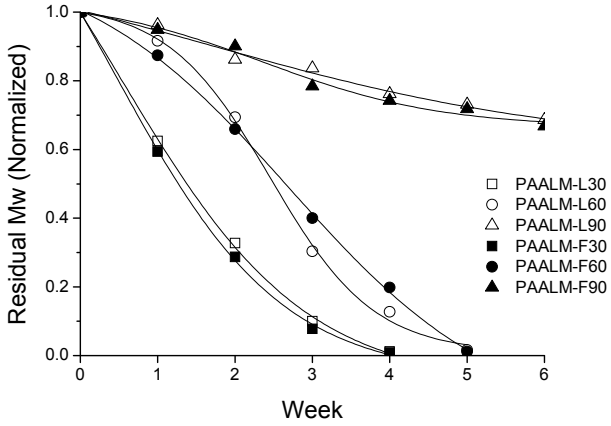


Figure 6.3. Molecular weight reduction of PAALM-L and PAALM-F copolyesters as a function of incubation time in PBS, pH 7.4 at 37 ºC.

The copolyesters PAALM-L60 and PAALM-F60, which are those with a more equilibrated compositions, were used to carry out the analysis of the released products by NMR and results are shown in Figure 6.4. Samples were incubated at 37 °C in deuterated water, and ^{1}H NMR signals arising from degradation soluble products were monitored. In the case of PAALM-L, the spectra clearly revealed the presence of ethanol in the solution just after two weeks of incubation as well as of free malic acid at the seventh week. In addition, signals from leucine methyl groups and from the polyester main chain were also detectable suggesting either oligomer solubilization or partial polymer solubilization. As expected, all signals increase in intensity at longer incubation times except those arising from oligomers which show a progressive diminution. For PAALM-F the hydrolysis proceeded similarly with methanol being the first product detected in the mother solution, and with signals arising from the degraded main chain products coming out after twenty weeks of incubation.

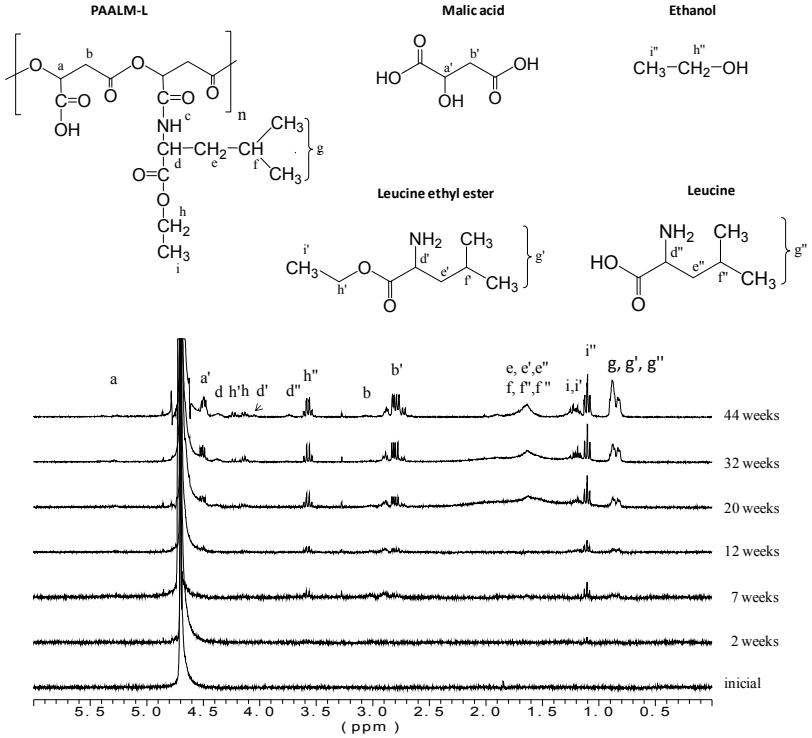
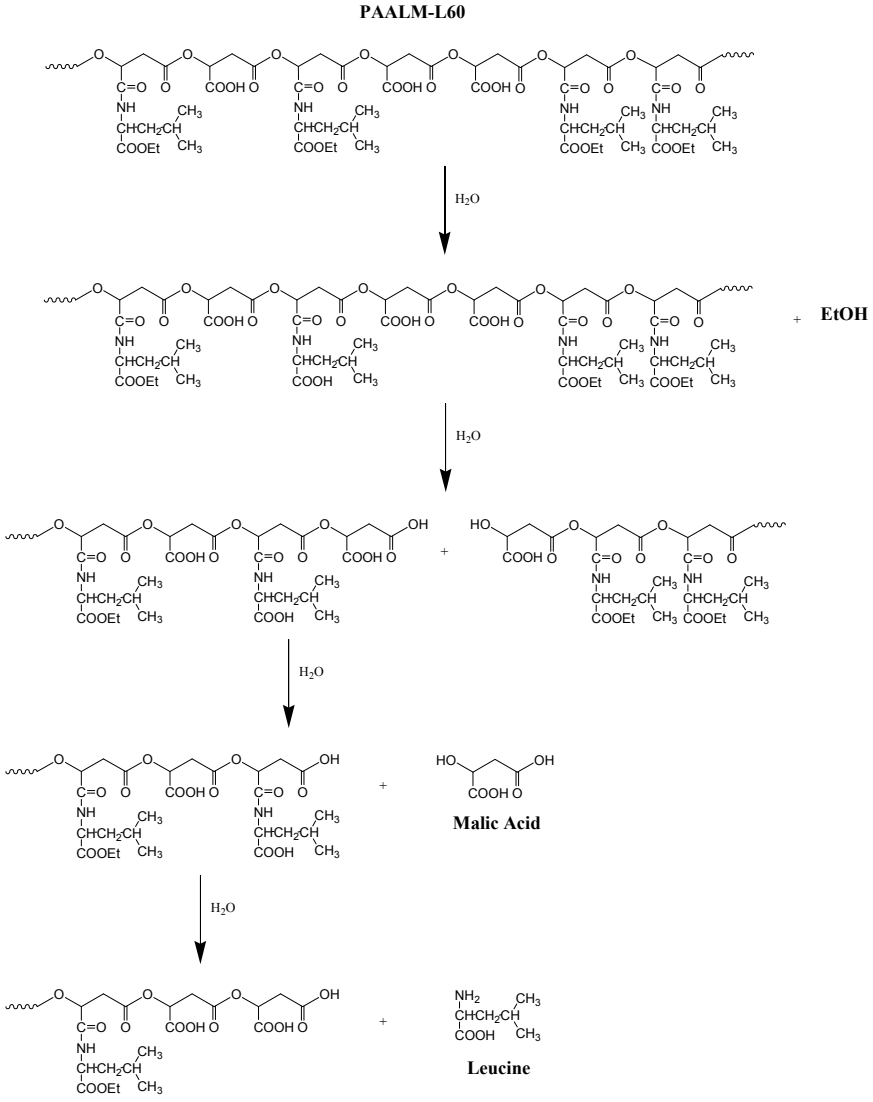


Figure 6.4. Evolution of the ^{1}H NMR spectra recorded from the water mother solution of the incubation of PAALM-L60 at 37 °C with time.

These NMR results supported by others previously obtained by us in the hydrolytic degradation of alkyl esters of PMLA suggest the hydrolytic mechanism for the copolyesters PAALM-L and PAALM-P depicted in Scheme 6.2. Hydrolysis starts with the cleavage of the amino acid ester groups with releasing of the corresponding alcohol, and continues with the splitting of the main chain at the ester linkages between non-amidated units with generation of oligomeric fragments and malic acid. Oligomers become solubilized or not depending on their amidation degree, and finally the hydrolysis of the main chain is completed with releasing of malic acid and free L-leucine or L-phenylalanine.



Scheme 6.2. Hydrolytic degradation mechanism of PAALM-L.

6.3.3. Nanoparticles formation

Spheric nanoparticles were prepared from copolymers PAALM-L and PAALM-F by applying the precipitation-dialysis method. An exploratory study of the influence of preparation conditions and copolymer conversion on the characteristics of the resulting nanoparticles was performed. Solutions at two different polymer concentrations (0.5 and 1.0 % w/v) in acetone, DMSO and methanol were tested. It was found that NPs were formed under all assayed conditions with a mean hydrodynamic diameter ranging between 70 and 230 nm (Table 6.2) depending on both the procedure applied and copolymer chosen. Firstly, the nanoparticle forming capability of copolymers with varying composition was examined, and results obtained with the PAALM-F series are illustrated in Figure 6.5. As it could be anticipated,[15] the morphology of the formed NPs varied with the ratio of hydrophobic to hydrophilic counterparts.

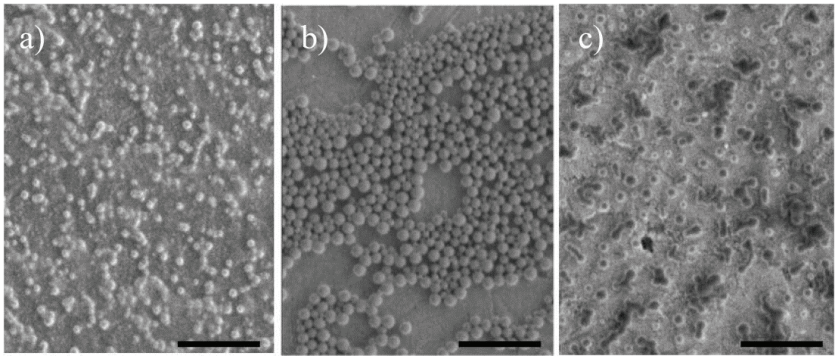


Figure 6.5. Nanoparticles of PAALM-F copolymers with different amidation degrees obtained from a 1.0 % (w/w) DMSO solution: a) PAALM-F30, b) PAALM-F60, and c) PAALM-F90. Scale bar: 1 μm.

Table 6.2. Mean hydrodynamic diameter of PAALM-L and PAALM-F nanoparticles obtained by the precipitation-dialysis method under different conditions.

	PAALM-L				PAALM-F			
Conv[a]	30	60		90	30	60		90
Conc[b]	1.0	0.5	1.0	1.0	1.0	0.5	1.0	1.0
DMSO	110±35	66±29	141±24	95±13	123±29	113±34	159±54	104±18
Methanol	126±27	76±38	199±36	109±31	145±18	137±37	222±42	-[c]
Acetone	173±20	156±33	227±19	139±12	180±24	180±41	231±22	147±35

[a] Degree of amidation (%).
[b] Initial concentration of the polymer solution (% w/v).
[c] Not measured in MeOH due to non-solubility of the copolymer in this solvent.

NPs prepared from 60 % amidated copolymers produced well defined nanospheres essentially exempted of amorphous material. On the other hand, solvent was the factor mainly deciding the nanoparticle size, the minimum values and dispersities being obtained with DMSO. This effect is well illustrated in Figure 6.6 for the case of PAALM-L copolymers. Results were very similar for the two series although particle sizes were much smaller when they were made from leucine amidated copolymers.

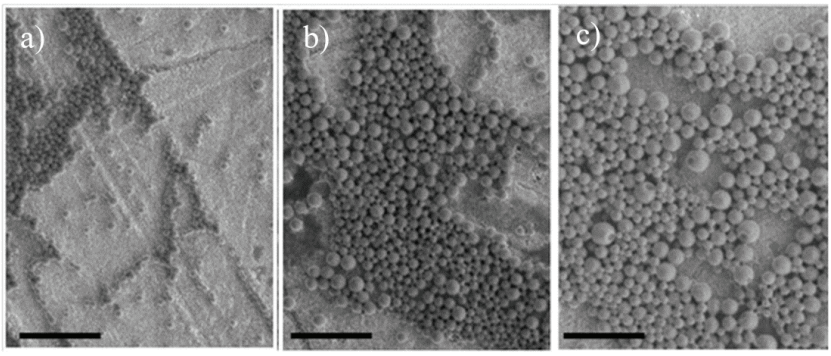


Figure 6.6. Nanospheres of PAALM-L60 prepared from 1 % (w/w) solution in different solvents: a) DMSO, b) methanol, and c) acetone. Scale bar: 1 μm.

6.3.4. Nanoparticle cytotoxicity

For its potential use as a biomaterial it was mandatory to evaluate the toxicity of nanoparticles. Thus, an in vitro study of PAALM-L60 and PAALM-F60 nanoparticles cytotoxicity on primary human glioma cell lines U-87MG, human non-small cell lung cancer, (metastatic in brain), CRL-5904, and invasive human breast carcinoma cell line MDA-MB-468, was performed. All cell lines presented a similar pattern with cell viability remaining near 100 % after 24 h of exposure to NPs in concentrations lower than 125 $\mu g \cdot mL^{-1}$. At higher concentrations there is a significant reduction in cell viability (Figure 6.7). Such concentration dependent cytotoxicity is related to the binding of polymer to membrane, followed by copolymer intrusion and irreversible membrane reorganization followed by a lytic event. Interactions between the copolymer hydrophobic microdomains and lipid bilayer membranes would explain membrane binding of the copolymers with subsequent membrane disruption.[154]

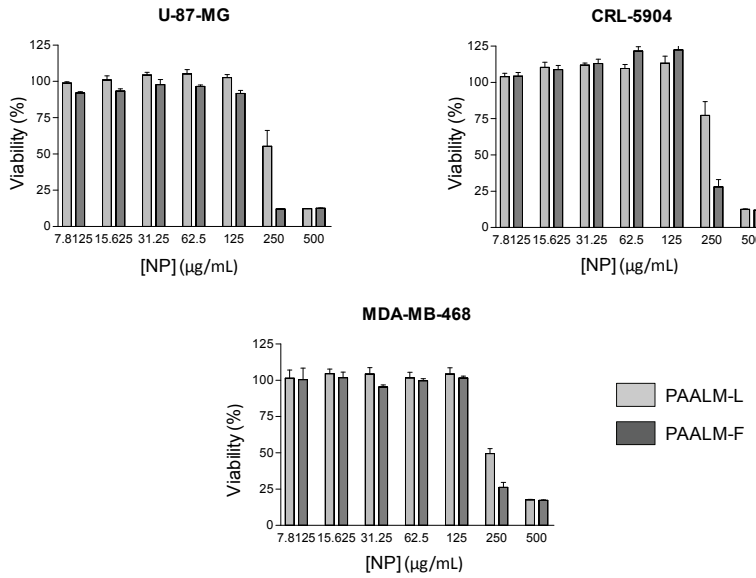


Figure 6.7. Cell viability of U-87-MG, CRL-5904 and MDA-MB-468 after 24 h of contact between polymer NPs of PAALM-L60 and PAALM-F60 and cells as a function of polymer concentration.

Ding and coworkers[59] observed that an increase in hydrophobicity and/or elimination of negative charges resulted in membranolytic activity of PMLA copolymers. They also found that the density of substituents on the polymer chain have an effect on membranolytic activity, observing a maximum efficacy at 40-60 % of substituents. The viability drop of U87MG after treatment with 0.5 % (w/v) of PAALM-L and PAALM-F60, could be also observed by optical microscopy and related with a significant change in cell morphology, which is more drastic after a treatment with 1 % (w/v) of NPs (Figure 6.8).

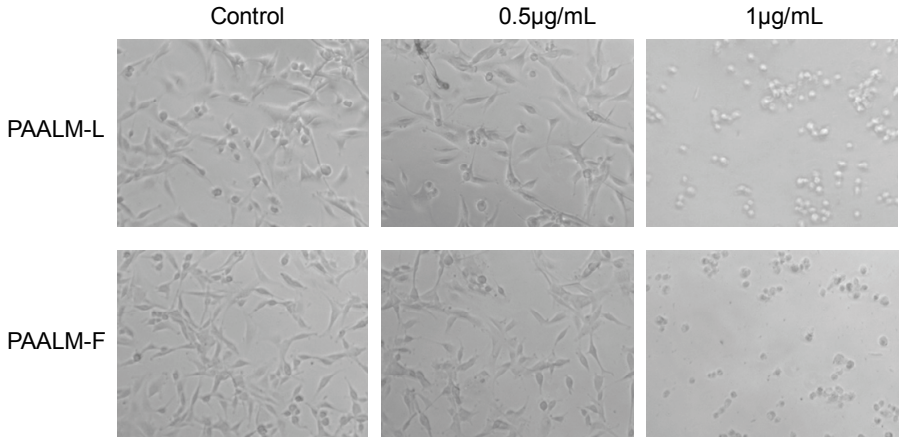


Figure 6.8. Cell morphology of cultured cells after 24 h incubation at 37 ºC for U-87MG cell line in the presence of nanoparticles of PAALM-L60 (top) and PAALM-F60 (bottom).

6.3.5. Drug encapsulation and in vitro release

PAALM-L60 and PAALM-F60 nanoparticles were chosen for studying the Temozolomide and Doxorubicin encapsulation and releasing. Drugs were encapsulated by the precipitation dialysis method, using methanol as the common solvent for both, drugs and polymer. The two copolymers showed a very similar behavior, with an entrapment

around 5 % (w/w) of TMZ and 8 % (w/w) for DOX. Although drug contents in the NPs could be considered acceptable, the encapsulation efficiency was low; DOX was entrapped about 28 % of its initial concentration and TMZ only around the half of such value (Table 6.3). The higher encapsulation efficiency for DOX can be explained by the fact that this compound could form ionic complexes with anionic polyelectrolytes.[103] Compared to our results obtained in the encapsulation of these drugs in poly(methyl malate) (PAALM-1) nanoparticles, encapsulated contents were doubled in the present case, which is likely due to the possibility of using a common solvent for both drug and polymer.

Table 6.3. Drug content and encapsulation efficiency of Temozolomide and Doxorubicin in PAALM-L60 and PAALM-F60 nanoparticles.

	TMZ		**DOX**	
	% Cont[a]	**% E.E.[b]**	**% Cont.[a]**	**% E.E.[b]**
PAALM-L60	4.9 ± 1.5	16.4 ± 5.1	8.5 ± 1.2	28.5 ± 4.2
PAALM-F60	4.2 ± 0.8	14.2 ± 6.2	8.4 ± 0.6	28.0 ± 2.1

[a] Percentage (w/w) of drug contained in the nanoparticles upon encapsulation.
[b] Percentage of initial drug that is encapsulated.

Cumulative Temozolomide release profiles from PAALM-L60 and PAALM-F60 nanospheres are presented in Figure 6.9. Since TMZ undergoes fast hydrolysis above pH 7.0 yielding AIC together with the methyldiazonium ion, which is the chemotherapeutically active molecule,[147,148] the analysis of the release profile under physiological conditions is complex. Thus, for a correct evaluation of *in vitro* TMZ release, it will be therefore necessary to quantify the delivery of both compounds, TMZ and AIC. The releasing profiles are very similar for the two copolymers, reaching almost 60 % of TMZ release after 3 hours of incubation and displaying a progressive decay concomitant to its hydrolytic degradation, after which TMZ was not longer detectable. Simultaneously, AIC concentration increased rapidly from the third hour

to the ninth, and more slowly afterwards. After 24 h of incubation, released TMZ was completely degraded. These results suggest that TMZ was firstly released form nanoparticles and then decomposed in the medium forming AIC with the consequent release of the methyldiazonium ion. A similar pattern of behavior was observed for the TMZ releasing from poly(methyl malate) nanoparticles.[153]

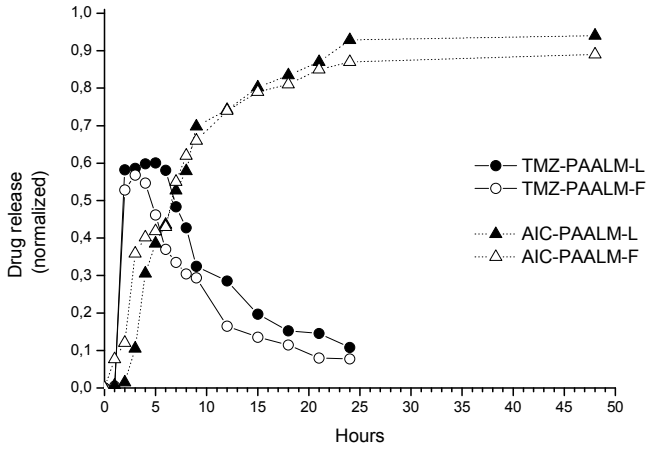


Figure 6.9. Profiles of TMZ *in vitro* release from PAALM-L60 and PAALM-F60 nanoparticles and formation of AIC from released TMZ at pH 7.4.

The release of DOX from PAALM-L60 and PAALM-F60 followed a similar profile in the two systems (Figure 6.10). The profiles were much simpler and revealed a much slower delivery rate than for TMZ; while only a few hours were required for the complete liberation of TMZ, the complete release of DOX took more than ten days. Such a comparative delay is likely to be due to the ionic interaction taking place between DOX and the free carboxylic groups present in the copolymers. Recently a similar behavior for the release of DOX from poly(γ-glutamic acid) particles has been reported[19], and it has been shown that DOX release was highly pH dependant; they found that at pH 2.2, which is below

PGGA pKa, the release reaches 60 % after 180 h of incubation whereas at pH 7.4 it was below 20 % after such time, a behavior very close to that observed in this work.

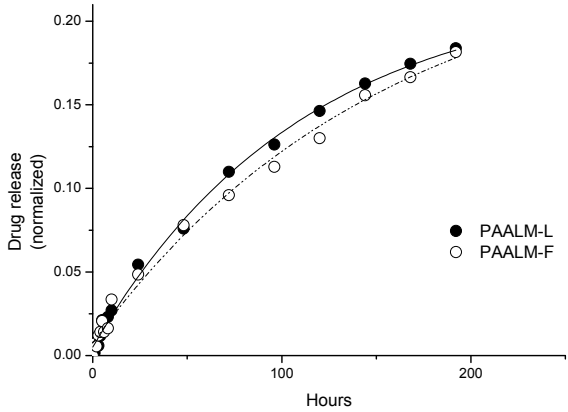


Figure 6.10. DOX *in vitro* release from PAALM-L60 and PAALM-F60 nanoparticles at pH 7.4.

6.4. Conclusions

Microbial polymalic acid partially amidated (30-90 %) with hydrophobic L-leucine (L) and L-phenylalanine (F) amino acid esters can be used to produce nanospheres with an average diameter ranging between 70 and 230 nm, depending on the kind of amino acid and the solvent used for preparation. The amino acid ester-grafted copolyesters are readily hydrolyzed in water at physiological conditions in times of weeks at a rate that decreases with the increasing degree of amidation. DOX and TMZ can be encapsulated in these nanoparticles and released upon incubation under physiological conditions. TMZ was released within a few hours with subsequent hydrolytic pH-dependent conversion into AIC, while DOX was steadily released in a time scale of days. The particles described did not show a sign of toxicity during the 24 hours of administration provided that NPs concentration is kept below 0.125 $mg \cdot mL^{-1}$.

7

Poly(β,L-Malic Acid)/Doxorubicin Ionic Complex: a pH-Dependent Delivery System

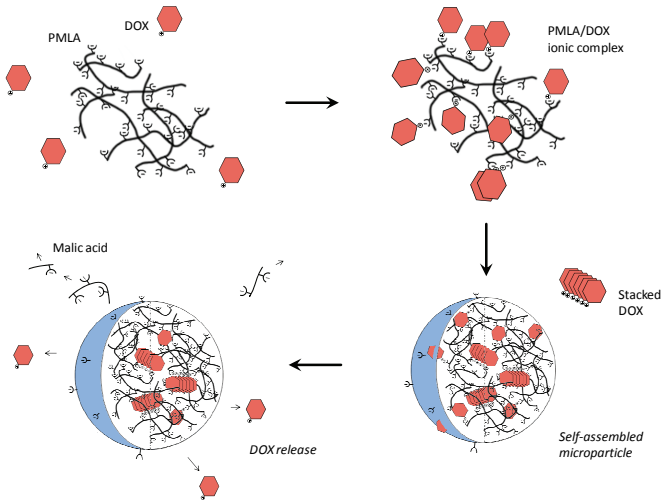
Aim and scope

The main drawback of anticancer chemotherapeutics is its high toxicity to both, cancer and healthy cells and tissues, especially Doxorubicin which can cause heart diseases. A strategy for improving therapeutic efficacy and limiting DOX toxicity has been to encapsulate the drug in carriers. In most of the drug delivery systems, drug is covalently attached or dissolved in the polymer matrix. But, for the case of polyelectrolytes, like PMLA, they are able to bind to an oppositely charged drug by electrostatic interactions forming a drug-polymer ents its ponic interactions are an effective way to bind a drug to a polymer; these

Ionic interactions are an effective way to bind a drug to a polymer; these interactions and therefore the formation of the ionic complex are pH dependant. As a result, drug-polymer ionic complexes are suitable candidates for drug delivery pH responsive systems. This chapter analyzes the capability of complexation between DOX and biotechnologically produced PMLA, for the generation of a pH-dependent drug delivery system.

Abstract

Poly(β,L-malic acid) (PMLA) was made to interact with the cationic anticancer drug doxorubicin (DOX) in aqueous solution to form ionic complexes with different compositions and an efficiency near to 100%. The PMLA/DOX complexes were characterized by spectroscopy, thermal analysis, and scanning electron microscopy. According to their composition, the PMLA/DOX complexes spontaneously self-assembled into spherical micro or nanoparticles with negative surface charge. Hydrolytic degradation of PMLA/DOX complexes took place by cleavage of the main chain ester bond and simultaneous release of the drug. In vitro drug release studies revealed that DOX delivery from the complexes was favored by acidic pH and high ionic strength.



7.1. Introduction

Biopolymer particles have found important applications as drug delivery systems and constitute an ideal option for encapsulating drugs since their activity, solubility, cell permeability and stability can be tuned by using polymers with appropriate chemical and physical properties.[80,103] Poly(β,L-malic acid) (PMLA) is a water-soluble, biodegradable, bioabsorbable and non-immunogenic polyester[141] that can be produced by either chemical synthesis[20,142] or by fermentation of certain microorganisms.[24] The properties and functionality of PMLA are adjustable by chemical derivatization; in fact PMLA has been esterified in different degrees to modulate the overall hydrophobicity of the polymer[47,95,153] or to introduce bioactive ligands.[50] PMLA ionizes readily in water (pK_a ~ 3.5) giving rise to a highly soluble polycarboxylate;[24] under these conditions it is able to be readily coupled with cationic compounds by stable ionic interactions.

Doxorubicin (DOX) is an anthracycline antibiotic that has been used for over 30 years as a potent chemotherapeutic antineoplastic agent to treat a wide spectrum of human cancers, especially breast cancer and lymphoma.[137] However, its therapeutic efficacy is limited because DOX long-term clinical use is compromised by the toxicity common to anthracycline drugs. Although encapsulation of DOX in lipid micelles has been used to overcome such shortcomings,[155,156] several essential attributes like drug release timing are difficult to control. New alternatives, like pH dependent release, are emerging with the purpose of optimizing the therapeutic action of DOX.[157-159]

The release of the drug in the free form from delivery systems is a prerequisite for the activity of most of the antitumor active agents. To attain a high antitumor activity along with a satisfactory cell-specificity, the characteristics of the polymer as well as the type of linkage between drug and carrier have to be properly chosen.[138] Chemical conjugation of DOX to poly(aspartic acid) has been done but it was revealed that was

actually the unconjugated DOX in the micelle which exerted the therapeutic effect.[160] Conjugation via acid-cleavable hydrazone bond has also been explored.[134,159] Other alternatives investigated has been the physical entrapment of DOX by nanoparticles[150,153] or micelles,[161] which offers advantages such as easy preparation and low cost but also suffers disadvantages such as limited drug loading and difficulty in drug release control.

Ionic interaction is an effective way to bind a drug to a polymer.[162] Such association is possible between polyelectrolytes and charged drugs and is termed as polymer/drug complexation.[103] The pH changes occurring within the body can address the response of the complex to a certain tissue or cellular compartment.[163,164] Carboxylic polymers, as it is the case of PMLA, are suitable candidates for pH responsive systems. These polyelectrolytes usually ionize in the 3 to 10 pH range to render polyanions able to bind substantial amounts of cationic drugs owing to their high negative charge density. DOX complexation with various polymeric systems like poly(acrylic acid),[165] poly(γ-glutamic acid)[103] and dextran sulfate[137] has been earlier reported. In this work we studied the complexation between DOX and microbially produced PMLA, as an easy and clean option for DOX encapsulation and pH-dependent release. To our knowledge it is the first time that polymalic acid is used as polyelectrolyte for direct coupling with a therapeutic drug. The well demonstrated capacity of PMLA to be bioassimilated along with its excellent hydrodegradability and biodegradability confer to this kind of complexes an exceptional interest for building pH-dependent drug delivery systems.

7.2. Experimental

7.2.1. PMLA/Doxorubicin ionic complexes synthesis

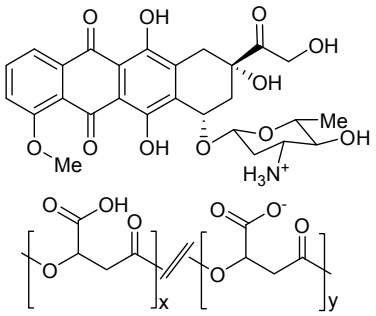
Ionic complexes of PMLA with DOX with different molar ratios were obtained by a simple and clean method. Briefly, to 1 mmol of PMLA dissolved in 4 mL of milli-Q water, pH ~ 6.0 (final pH ~ 4), 0.1, 0.25 or 0.5 mmol of DOX dissolved in 2 mL of the same solvent were added dropwise under magnetic stirring, and left to react overnight in the dark to prevent DOX photodegradation. Non-attached DOX was removed by extensive dialysis against deionized water for 48 h using a cellulose membrane of 8 kDa molecular weight cut-off. The PMLA/DOX ionic complexes precipitated from solution and were lyophilized for recovery and storage. The complexation degree attained was determined by ^{1}H NMR.

7.3. Results and discussion

7.3.1. Synthesis and characterization

Poly(β,L-malic acid) (PMLA) ionizes readily in water giving rise to a highly soluble polyanion. The pK_a of biologically produced PMLA has been determined to take values within the 3.4-3.6 range so it is extensively charged under physiological conditions.[24] On the other hand, DOX is a positively charged amphoteric drug containing one protonable amino group in the sugar moiety with a pK_a=8.6 and two deprotonable phenolic groups in the aglycone part of the molecule; thus an equilibrium exists between the positively charged, negatively charged and neutral species of DOX depending on pH. In the 0-6 pH range, the amino group in DOX is protonated as NH_3^+ which makes possible its electrostatic binding to negatively charged PMLA;[103] in fact, non-stoichiometric ionic complexes with polymer/drug molar ratios of 10:1, 4:1 and 2:1 were successfully obtained by precipitation upon mixing the

two components in an aqueous medium that was initially set at pH 4.0 (Scheme 7.1). Complexes precipitated from the solution displacing the equilibrium towards their continuous formation.



Scheme 7.1. Non-stoichiometric ionic complex PMLA/DOX.

The complexes were formed with high efficiency for whichever composition with more than 90 % of the added drug becoming ionically coupled to PMLA (Table 7.1). Such loading efficiency was up to three times higher than that attained in the physical entrapment of DOX in esterified PMLA derivatives using the emulsion-evaporation[153] or precipitation-dialysis methods.[150] The partial charge neutralization taking place upon complexation resulted in the instantaneous precipitation of a reddish product in the form of particles. For PMLA/DOX-(10:1) and PMLA/DOX-(4:1), particles remained in a colloidal suspension, while those made of PMLA/DOX-(2:1) tend to aggregate and settle down onto the bottom of the container.

Table 7.1. Coupling reaction characterization.

	Feed Ratio mol:mol	Complex Composition (mol:mol)	Efficiency (%)	Yield (%)	M_w (g mol^{-1})
PMLA	-	-	-	-	32,000
PMLA/DOX-(10:1)	1:0.10	1:0.09	99	85.7	29,300
PMLA/DOX-(4:1)	1:0.25	1:0.23	92	83.3	29,800
PMLA/DOX-(2:1)	1:0.50	1:0.46	92	80.5	29,600

Since aqueous PMLA/DOX mixtures evolve with immediate precipitation of the complex, the occurrence of interactions between the two components in solution is difficult to investigate. To approximate the question ^{1}H NMR spectra were taken from aqueous solutions of malic acid/DOX mixtures over a wide range of composition ratios, and compared to the spectra of the pure components. No differences were detected for any composition, which can be taken as indicative of complete absence of specific interaction between MLA and DOX in aqueous solution (^{1}H NMR spectra shown in the Support Information, appendix 2, Figure A2.1).

Conversely the formation of the ionic PMLA/DOX complex in the solid state could be ascertained by infrared spectroscopy. The FT-IR profiles registered from the 2:1 complex and from its components, both separately and physically mixed are compared in Figure 7.1, and characteristic absorption bands are listed in Table 7.2. Main changes detected in the spectrum of the ionic complex compared to that registered from the physical mixture or PMLA and DOX are the disappearance of the 3528 and 1521 cm^{-1} bands, as well as the decrease in intensity of the 869 and 802 cm^{-1} bands; all these bands are associated to the DOX NH bond vibrations (stretching, bending or wagging), and their absence indicates that the DOX amine group must be directly involved in the association of the drug with the polyacid, most probably through

ionic interaction. Similar changes in the DOX infrared spectrum were reported in the work of Kayal and Ramanujan,[166] in which they demonstrated the attachment of DOX to PVA coated iron oxide nanoparticles via amine-hydroxyl interactions. In order to support our interpretation the solid product resulting from evaporating an aqueous solution containing equimolar amounts of malic acid and DOX was also examined by FTIR. The spectrum from the compound showed also a large decreasing of the 1521 cm^{-1} DOX band according to what should be expected for the interaction of the amine group of DOX with the carboxylic group of malic acid (Figure A2.2).

Table 7.2. FT-IR of PMLA and DOX, their physical mixture (2:1), and their ionic complex (2:1).

Absorption bands (cm^{-1})				Assignment
PMLA	**DOX**	**PMLA+DOX**	**PMLA/DOX**	
	3528	3528		*ν*(O-H) free
	3316	3316		*ν*(H-O) bonded
	3160-2300	3160-2300		*ν*(NH_3^+)
	2897	2897		*ν*(C-H)
1730	1730	1730	1730	*ν*(C=O)[a]
	1615	1615	1615	*ν*(C=O)[b]
	1580	1580	1580	*ν* (C=C)
	1521	1521		δ(N-H)
	1413	1413	1413	δ(CH)
	1283,989	1283,989	1283,989	*ν*(C-O-C)
1157			1157	*ν*(O-C-C)
	1071	1071	1071	*ν*(C-O)
1044				*ν*(C-O), *ν*(C_α-C_β)
	869/802	869/802	869	ω(N-H)

[a] Band from ketone in DOX.
[b] Bands from quinone carbonil associated by intramolecular hydrogen bonds.

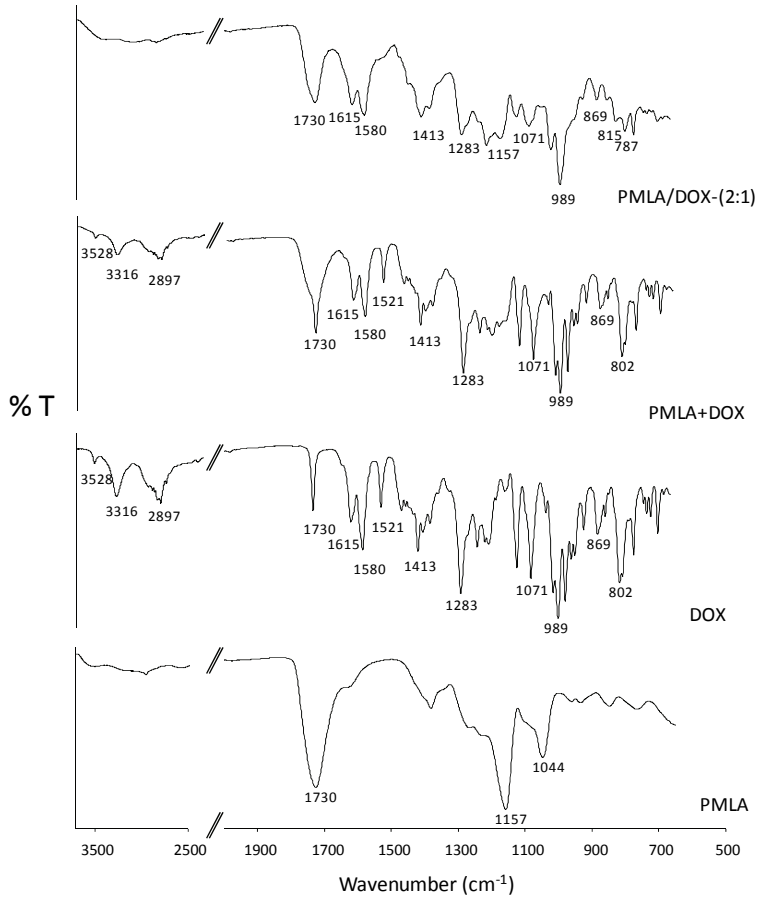


Figure 7.1. FTIR spectra of the PMLA/DOX-(2:1) complex, the PMLA+DOX (2:1) physical mixture, DOX, and PMLA.

The amount of drug bound to the polymer was quantified by NMR spectroscopy. The ^{1}H NMR spectra of complexes and their components are compared in Figure 7.2. Calculations for drug content were done using the signal at 1.55 ppm arising from the methyl group attached to the pyrano moiety in DOX and the signal located at 5.6-5.9 ppm, which arises from the PMLA main chain CH_{α} and one CH of DOX. The PMLA/DOX molar ratios in the complexes as determined by this method are listed in Table 7.1, which were found to be very close to the feed.

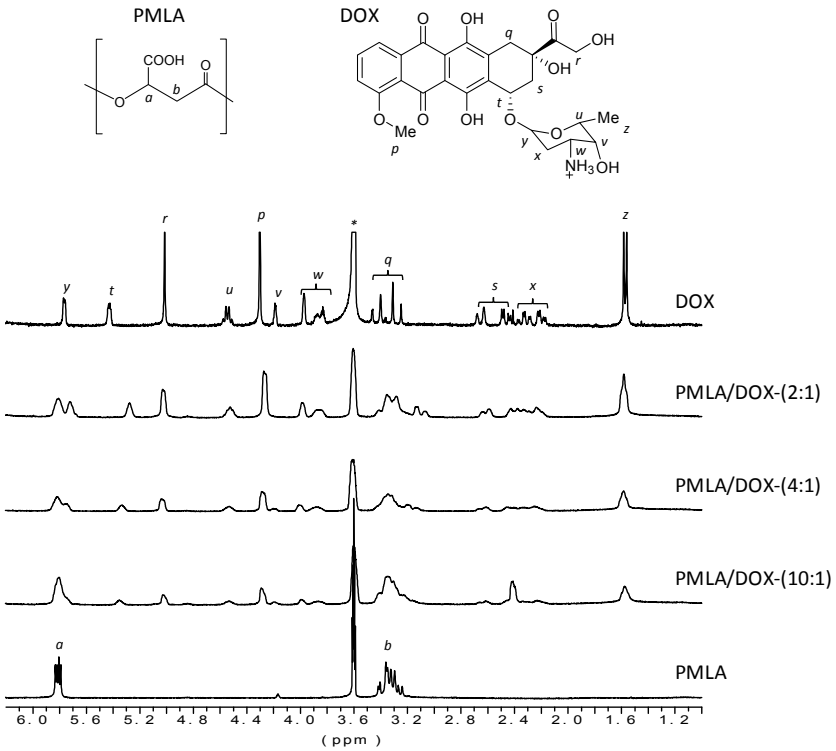


Figure 7.2. ^{1}H NMR spectra of PMLA, DOX and PMLA/DOX ionic complexes.

7.3.2. Thermal characterization

A DSC analysis of the complexes was carried out in order to appraise their thermal behavior. The DSC traces of PMLA, DOX, their physical mixture with a 2:1 ratio, and the ionic PMLA/DOX-(2:1) complex are compared in Figure 7.3. The DSC trace of PMLA displayed a wide melting peak at 210-215 °C, indicative of the semicrystalline nature of this polymer, and the trace of DOX presented a well-defined melting peak at 231 °C characteristic of highly crystalline material. Conversely the trace registered from the PMLA+DOX physical mixture is essentially similar to that of PMLA with the melting peak broadened and displaced downwards due to the presence of DOX, which presumably was

dispersed in molten PMLA. On the contrary, the heat exchange detectable in the trace of the PMLA/DOX-(2:1) complex was almost imperceptible indicating that crystallinity of PMLA was largely suppressed due to the ionic interaction taking place between the drug and the polymer. Furthermore, the total absence of the characteristic DOX melting peak on the complex may be taken as demonstrative of that no pure DOX precipitated separately during complexation.

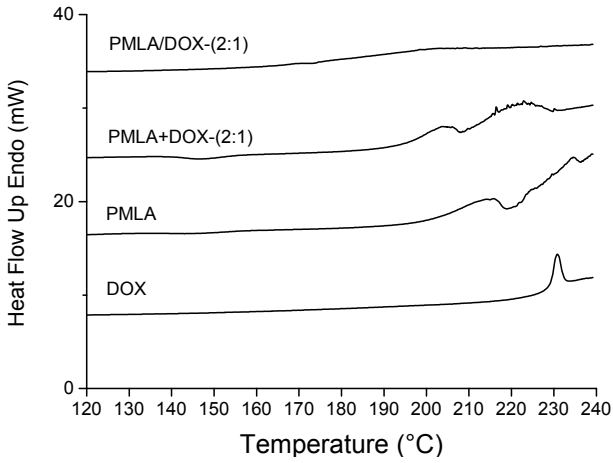


Figure 7.3. DSC traces of the PMLA/DOX-(2:1) complex, the PMLA+DOX-(2:1) physical mixture, PMLA and DOX.

The thermal stability of DOX/PMLA complexes was evaluated by thermogravimetry. The TGA traces of the complexes and their components are compared in Figure 7.4. The initial weight loss of about 5 % seen on the TGA traces of samples containing PMLA is attributed to their moisture content. More than 90 % of weight loss of PMLA occurred over the 200-250 °C range, which is consistent with the degradation signal that is observed by DSC overlapping the melting of the polymer. On the other hand, DOX degradation was found to happen along three steps that started at 237, 320 and 400 °C, respectively, and that entailed a

total weight loss of 50 % of the initial mass. The complexes also presented a degradation in three steps but at lower onset temperatures and entailing smaller weight losses and that are depending on composition. Remaining weights after heating at 800 °C were 20, 27 and 38 %, for 10:1, 4:1 and 2:1 PMLA/DOX complexes respectively. In the three cases thee second degradation step happened about 325 °C with a weight loss corresponding to the DOX degradation second step.

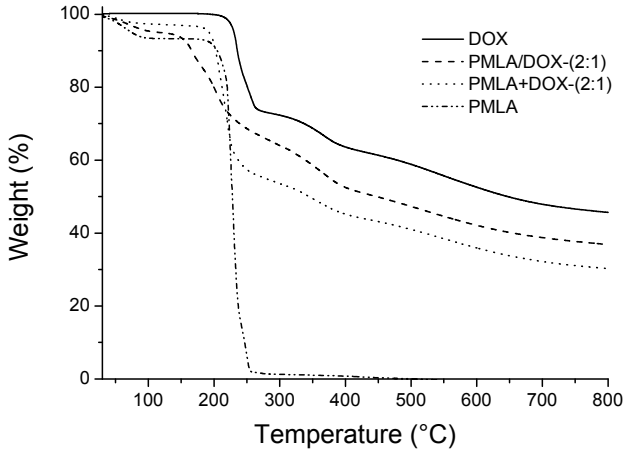


Figure 7.4. DSC traces of the PMLA/DOX-(2:1) complex, the PMLA+DOX-(2:1) physical mixture, PMLA and DOX.

7.3.3. Particle formation and characterization

Particles formed during drug-polymer complexation were examined by scanning electron microscopy. Since the ionic complexes are amphiphilic they are expected to self-assemble in water to form spherical particles, presumably with the hydrophobic DOX-coupled PMLA units integrating the inner particle core and the remaining ionized polymer segments preferentially located near to the surface. In fact,

individually dispersed microspheres for 10:1 and 4:1 complexes and aggregated nanospheres for 2:1 complex were observed (Figure 7.5).

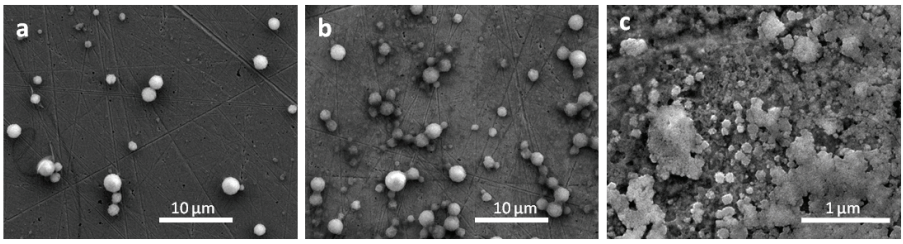


Figure 7.5. SEM micrographs of PMLA/DOX complex particles. a) PMLA/DOX-(10:1), b) PMLA/DOX-(4:1), and c) PMLA/DOX-(2:1).

The hydrodynamic average diameter of microparticles determined by DLS was around 1.5 µm while in nanospheres it was ten times smaller, *i.e.* 150 nm approximately. The result is comparable to that reported for the chemical conjugation of DOX with poly(α-aspartic acid) which changed the initial hydrophilic units of the polyacid into hydrophobic blocks by ionic coupling with the drug. The coupled DOX will self-assembly to form a hydrophobic micelle core.[160] A somewhat simple model illustrating the possible structure of the particles is depicted in Figure 7.6. The role of the ionic interactions as the main cohesive forces operating in the particles was evidenced by appraising the effect that the ionic strength exerts on their stability. In fact, decomposition of the complex with subsequent liberation of DOX to the medium took place in particles suspended in NaCl aqueous solution as soon as the salt concentration came up around 0.1M (Figure 7.7).

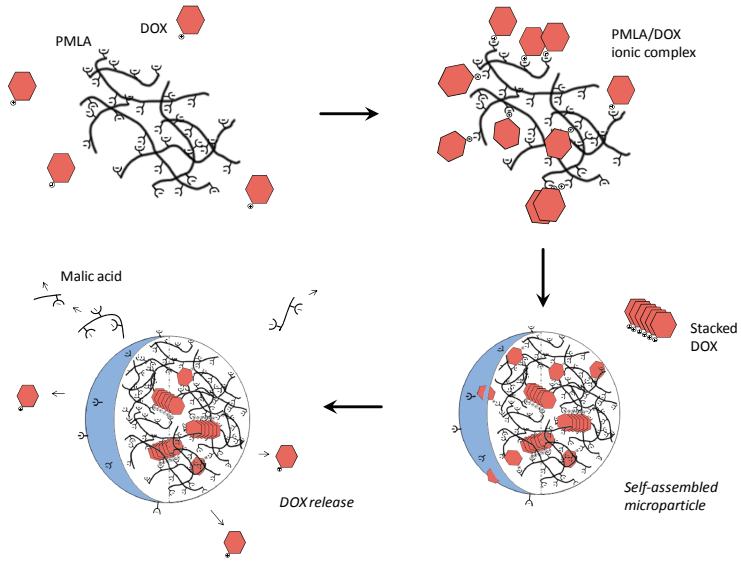


Figure 7.6. Schematic model of the interaction mechanisms operating in the PMLA/DOX particle formation and drug loading and release.

The surface charge of the particles, reflected by their ζ potential, had approximately the same negative value for the three complexes confirming the outer location of the non-coupled carboxylic groups (Table 7.3). These values are significantly lower than that reported for neat PMLA.[167] Although positively charged particles are preferred for cell uptake, it is known that negative particles are also able to undergo endocytosis by adsorption at the positively charge cell sites.[168] In fact a combination of factors including size, shape and surface chemistry is actually governing cellular uptake. Regarding the zeta potential, it is accepted that particles may be internalized if values of ζ are within the range of tens of -mV and + mV.[169]

Table 7.3. PMLA/DOX ionic complex particles characterization.

	Particles	State	Size[a] (nm)	Pd.I.[b]	ζ potential (mV)
PMLA		Solution			-22.9 ± 1.7
PMLA/DOX-(10:1)	micro	Suspension	1613	0.544	-26.0 ± 5.1
PMLA/DOX-(4:1)	micro	Suspension	1496	0.447	-28.0 ± 4.8
PMLA/DOX-(2:1)	nano	Aggregate/Precipitation	150	0.114	-26.9 ± 4.0

[a] Hydrodynamical average diameter.
[b] Polydispersity index of particle size.

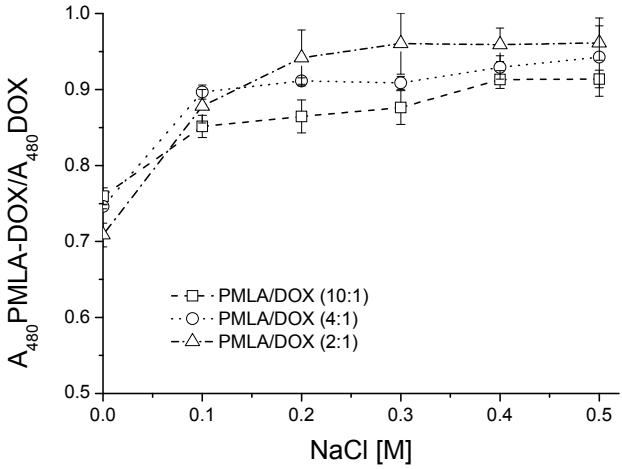


Figure 7.7. Normalized absorbance of DOX released from PMLA/DOX complexes as a function of NaCl concentration.

7.3.4. Hydrolytic degradation mechanism

The hydrolytic degradation of the complexes was followed by ^{1}H NMR analysis of the products released to the incubating medium (Figure 7.8). The supernatant of PMLA/DOX-(10:1) incubated at pH 7.4 and 37 ºC showed signals arising from the methylene groups of PMLA after 3 days of incubation which are attributed to the presence of partially solubilized large size oligomers. The presence of short oligomers was

later evidenced by the appearance of a signal at 2.90 ppm arising from the β-CH_2 of the PMLA end-chain unit. The intensity of this signal increased continuously along the two first weeks of incubation to be finally replaced by the 2.70 ppm methylene signal from free malic acid. Weak signals characteristic of DOX were detected from the third day of incubation (Figure 7.8a) but their intensity remained essentially unchanged due to the instability of this compound in the incubating medium. In fact it has been reported that DOX in aged aqueous solution tends to form clusters with water included between the stacked aromatic sheets which are excluded from solution in form of gel.[170,171] We have experimentally checked this behavior by NMR and results have been included in the Annex section as Figure A2.3. The NMR analysis of PMLA/DOX-(2:1) incubated under similar conditions afforded the same signal changing pattern but with lower peak intensity and a delay of one week in the appearance of new signals, in agreement with what should be expected from its lower content in non-complexed PMLA residues.

The ^{1}H NMR spectra recorded from the incubation medium at pH 5.0 were more difficult to analyze due to the fact that the signals of the citric acid used for pH buffering overlapped with the methylene signals of the free malic acid and terminal malate units. Nevertheless, a close inspection of the spectra shown in Figure 7.8b revealed that they followed an evolution pattern similar to that observed at pH 7.4 but at a higher rate. Degradation of PMLA/DOX-(10:1) at pH 5.0 clearly showed signals from terminal β-CH_2 of the PMLA chain after 3 days of incubation, and at difference with what happened at pH 7.4, signals arising from the CH end groups were also detected at this time (Figure 7.8b). ^{1}H NMR spectra recorded at different pH for PMLA/DOX-(2:1) showed slighter differences than for PMLA/DOX-(10:1). Although less soluble products and a lower rate of disappearance of the main chain signals were observed in this case, DOX signals started to be observed for both complexes at the third day of incubation.

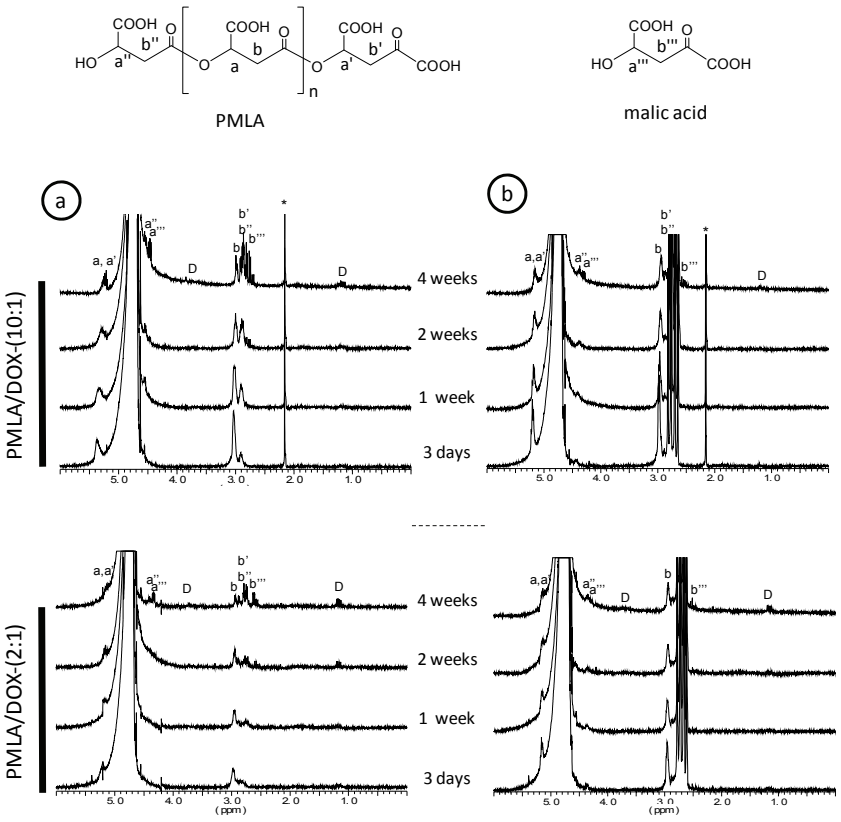


Figure 7.8. ^{1}H NMR spectra of the degradation media of ionic complexes PMLA/DOX-(10:1) and PMLA/DOX-(2:1) at different pHs: a) 7.4 and b) 5.0. D = DOX, * solvent traces.

7.3.5. In vitro drug release

It is known that pH and ionic strength play a key role in the liberation of drugs that are immobilized to a polymer matrix by ionic coupling interactions. Drug release from PMLA/DOX complexes was assessed as a function of releasing media conditions concerning pH and ionic strength. The physiological pH 5.0 and 7.4 were assayed; pH 5 emulates inside mature lysosome environment whereas pH 7.4 imitates plasma conditions. The cumulative DOX releasing profiles observed for the three studied complexes as a function of time are comparatively

plotted in Figure 7.9a for the two assayed pH values. DOX release appeared to be markedly pH dependent since at pH 5 the delivery rate was four times faster than at pH 7.4 regardless complex composition. In fact, the cumulative amount of DOX released after 25 days of incubation at pH 5 was 35-40 % of the initial load whereas at pH 7.4 it hardly reached 15 %. Furthermore, the cumulative release at pH 7.4 tends to a stabilized on a plateau while at pH 5 the release rate was almost constant along the whole incubation time. A moderate burst is noticed at either pH and at any ionic strength. The small fraction of DOX initially delivered at high rate would be that located nearly the surface. The release of inner located drug will be somewhat depending on polymer degradation and its delivery rate is expected to decay. Both H-bonding PMLA/DOX interactions and π-π stacking DOX/DOX interactions would be responsible for the further delayed or even incomplete drug release observed in these complexes.

The pH dependence observed for the PMLA/DOX complexes is certainly a relevant result since drug release during blood transport would be prevented whereas drug discharge would take place at the target compartment where the pH is expected to be around 5.0. Tumor tissue has an extracellular pH of 6.5-7.2, slightly lower than the 7.4 value present in normal tissues, whereas inside the lysosomes, where the microparticles would access via phagocytosis, the pH is 4.5-5.0. Furthermore the hydrolytic enzymes therein present could enhance polymer degradation and therefore drug release. Since residence times of a few hours should be expected for a carrier in the blood stream, DOX losses from microparticles made of PMLA/DOX complexes are expected to be negligible during transport. This is a highly appreciated advantage for cytotoxic drugs like DOX, although only an efficient delivery at the site of action will allow to taking full benefit.[103]

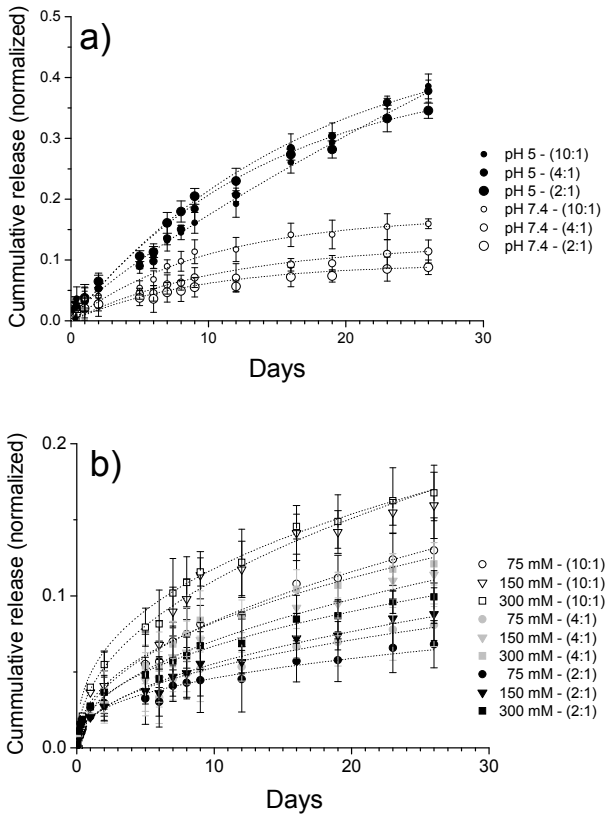


Figure 7.9. Doxorubicin release from PMLA/DOX ionic complexes as a function of pH (a) and ionic strength at pH 7.4 (b).

Regarding compositions it was observed that release rate differences between complexes at pH 5 were small, whereas they were significant at pH 7.4. Apparently the DOX release rate is dependent on the complex composition provided that incubation is performed under neutral conditions. Probably such a different behavior is related to the effect of pH on degradation rate of PMLA. Also the effect of the ionic strength on DOX release was studied at pH 7.4 for *I* values of 75, 150 and 300 mM (Figure 7.9b). A clearly enhancing effect of the ionic strength on the DOX release rate was observed for the three complexes, although in not so much extent as for pH changes. The maximum release rate

observed at pH 7.4 was for the PMLA/DOX (10:1) complex at 300 mM ionic strength; the drug accumulated in the incubation medium after 26 days of residence was 17 % of the initial loaded amount.

The release kinetics of DOX physically entrapped in nanoparticles made of PMLA derivatives has been recently studied.[150,153] DOX release rates at pH 7.4 from PMLA/DOX complexes measured in this work are proven to be up to three times slower than the release reported for physical entrapped DOX (Table 7.4). On the other hand, in the work of Manocha et al. on the ionic complex made of poly(γ-glutamic acid) and DOX,[103] the release of the drug was reported to take place initially following a profile similar to that reported for physically entrapped systems but reaching a plateau at the end of the incubation period. This is according to expectations since release of the physically entrapped drug is mainly depending on diffusion/degradation factors whereas the releasing from ionically coupled systems, as it is the case of polyacid/DOX complexes, is largely determined by the environment conditions required to cleave the ionic link between the drug and the polymer.

Table 7.4. Comparative of DOX release by different PMLA-based delivery systems.

	Method of encapsulation	DOX content (w/w %)	Release at 24 / 240 h (%)	Reference
PMLA/DOX (10:1)	Simple mix - precipitation	29.7 ± 2.4	2.7 / 8.3	This work
PMLA/DOX (4:1)	Simple mix - precipitation	107.6 ± 1.9	2.1 / 7.1	This work
PMLA/DOX (2:1)	Simple mix - precipitation	234.0 ± 3.1	2.0 / 5.4	This work
PGGA	Simple mix - precipitation	99.0 ± 0.06	8.5 / ~18	Manocha *et al.*, 2010[2]
PAALM-1	Emulsion - solvent evaporation	4.2 ± 0.6	14.3 / 27.4	Lanz *et al.*, 2011[9]
PAALM-L60	Precipitation - dialysis	8.5 ± 1.2	5.2 / 30.2	Lanz *et al.*, 2012[19]

7.4. Conclusions

Result presented in this work show that the biopolymer PMLA can be used as a carrier for targeted delivery of DOX, a hydrophobic drug widely used in cancer therapy. In aqueous solution ionized PMLA interacted efficiently with DOX to form stable ionic complexes. The complexes tend to self-assemble in micro or nanospheres according to their polymer/drug ratio. These particles underwent hydrolysis at a rate that was dependent on pH and complex composition. The ability of PMLA/DOX complex particles to operate as a pH-sensitive drug delivery system has been evidenced. The DOX release rate from the complexes was enhanced by decreasing pH and, in less degree, by increasing the ionic strength of the medium. Furthermore the release rate could be modulated by adjusting the composition of the complexes. It can be concluded therefore that PMLA/DOX complexes afford a good potential for drug delivery in cancer therapy.

8

Modification of Microbial Polyglutamic and Polymalic Acids Via *Click* Chemistry: Nanoparticle Formation and Drug Encapsulation

Aim and Scope

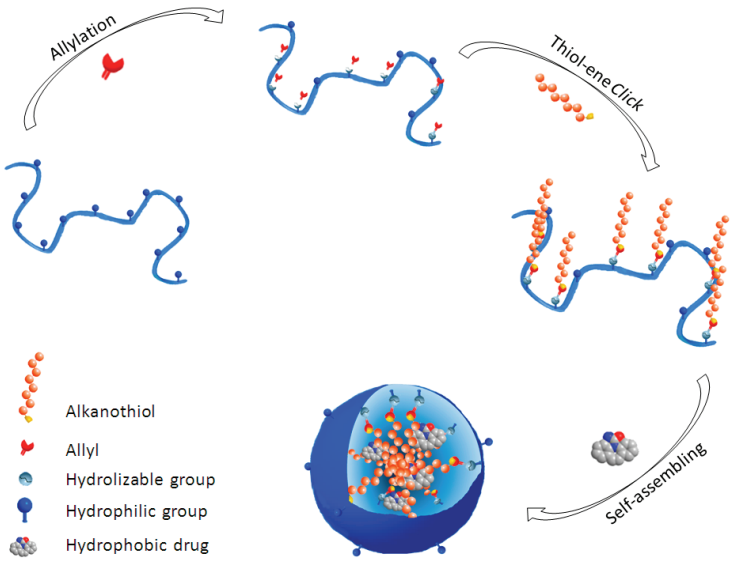
Advances in drug delivery systems and in the understanding of cellular and molecular biology have provoked an increasing need for new materials with better defined structures or functionalities; however, the preparation of such materials imposes major synthetic challenges.

Click chemistry provides alternatives to conventional strategies used for post-polymerization modification; a two step functionalization of PMLA is possible under the mild conditions used in modern click conjugation strategies. Thiol-ene chemistry is a metal catalyst-free approach, and has been demonstrated to be a powerful and versatile method for site specific functionalization.

In this section, we studied aliphatic long chains grafting through thiol-ene click reactions, in order to obtain aliphatic comb-like polymers for nanoparticle formation and drugs encapsulation; as a first approach to a multifunctional modification.

Abstract

Comb-like polymers were obtained by esterification of the carboxylic side groups of microbial poly(β,L-malic acid) (PMLA) and poly(γ,DL-glutamic acid) (PGGA) by grafting aliphatic long chains trough a two steps method involving UV-initiated thiol-ene *click* reactions. Amphiphilic derivatives were capable to form self-assembled nanoparticles in the range of 80-240 nm of diameter. Incubation under physiological conditions leads to the hydrolysis of the polymers, by the cleavage of the lateral ester bonds and later scission of the main polyester or polyamide chain. The model drugs, Theophylline and Carbamazepine were encapsulated with efficiencies up to 38.0 % with much better results for the later. Drug release from nanoparticles incubated under physiological conditions occurred with a burst effect and complete discharge of the drug in 24 h. Release profiles from drug-loaded films suggest that drug release is driven by a diffusion process.



8.1. Introduction

Biodegradable polymers are currently of great interest for their use in temporal biomedical applications like chirurgical sutures and drug delivery systems. Among them, biopolymers of natural occurrence, or biotechnologically produced polymers stand out because they must be bioassimilable and biocompatible since they are produced from bioorganic molecules.[80,141,172] Polyamides and polyesters, like poly(γ-glutamic acid) (PGGA) and poly(β,L-malic acid) (PMLA) respectively, are produced by fermentation with bacteria and fungi, respectively, and are excellent candidates as biodegradable materials for medical applications.

PGGA is one of the three poly(amino acid)s present in nature that can be produced by several species of bacteria of the genus *Bacillus*, classified as GRAS (Generally Regarded As Safe) by the US Food and Drug Administration.[88] PGGA is a water-soluble polyamide that degrades into glutamic acid which is an essential substance to humans.[82,173] The outstanding biodegradability and biocompatibility of poly(amino acid)s have attracted considerable attention and have made them object of much attention for their potential utilization in the fields of drug delivery system (DDS), tissue engineering, and other biomedical applications.[174,175] PGGA and its derivatives are considered promising materials that distinguish by being able to display functional properties due to the presence of the carboxylic side group attached to the polyamide main chain.

PMLA is naturally produced by myxomicetes and filamentous fungi. It is a water soluble polyester very prone to undergo hydrolysis.[24] PMLA is also a promising building block for the design of efficient drug delivery systems because its excellent biodegradability and biocompatibility and because its degradation produces easily metabolized L-malic acid.[26] PMLA and its derivatives have been used as platform in the synthesis of nanocarriers for drug delivery[18,48,53,56] or as a

constituent in macromolecular conjugates bearing several functionalities to treat human brain and breast tumors in mouse models.[50,176]

Nowadays, biodegradation and bioresorption of any high molecular weight material for biomedical applications is considered as a prerequisite for its use in human therapy.[18] This has stimulated the modification of naturally occurring biopolymers and the development of new synthetic ones.[20,21] Both PGGA and PMLA bear pendant carboxylic groups which make their derivatization feasible for the modulation of polymer hydrophobicity and properties [51,77] and for the introduction of bioactive ligands required for the stable association with drugs and proper release.[78,167]

The concept of postpolymerization functionalization strategies introduces techniques based on *click* chemistry.[104] *Click* chemistry embraces a number of simple, modular, and highly specific chemical reactions featuring high yields under mild conditions.[111] In this context, thiol-ene chemistry has most of the attributes of *click* chemistry. Furthermore, it is a metal catalyst-free approach that has been proved to be in both, its radical and base/nucleophilic forms, a powerful and versatile method for site specific functionalization as well as a conjugation tool convenient for a wide range of applications, including polymer functionalization, dendrimer synthesis and nanoimprinting.[107,177] There are several features associated with the thiol-ene reaction that makes it particularly attractive, facile and versatile process; *i)* it results in a stable linkage, *ii)* it exhibits minimal cross-reactivity with other functional groups, *iii)* reaction arrives close to completion, *iv)* resulting products are free of appreciable amounts of impurities and *v)* it proceeds under mild conditions.[108]

In this work we take benefit from the functionality of PGGA and PMLA for the construction of amphiphilic comb-like polymers for their application as DDS. Amphiphilic block or graft copolymers consisting of hydrophilic and hydrophobic segments are capable of self-assembling in

aqueous solutions to form micro or nanoparticles.[53,86] DDS based on polymer particles are clearly advantageous because: a) particle size and surface can be engineered to achieve passive or active drug targeting, b) drugs can be incorporated without chemical reaction, c) drug activity is optimally preserved during its transportation to the site of action, d) site-specific targeting can be achieved by attaching targeting ligands, e) formulation can be delivered trough different routes of administration and f) controlled drug release can be achieved.[2,80,116,117]

We present a two-step modification of PGGA and PMLA as a first approach for PGGA and PMLA functionalization through thiol-ene reactions. In the first step the allyl group is introduced by direct esterification, which leads to a double-bond functionalized polymer with potential for orthogonal, specific and multifunctional modification.[178] In the second step aliphatic long chains are grafted in order to obtain the comb-like architecture required for nanoparticle formation and drug encapsulation. Drugs used for encapsulation and drug delivery assays were Theophylline and Carbamazepine which are considered as hydrophilic and hydrophobic drug models, respectively.

8.2. Experimental

8.2.1. Esterification reactions

Esterification of PGGA with allyl bromide was carried out following the procedure described elsewhere for esterification of PGGA with alkyl-bromides.[179] Briefly, $NaHCO_3$ (525 mg) was added to a solution of 200 mg of PGGA in 20 mL of *N*-methylpyrrolidone (NMP) heated at 60 °C. Allyl bromide was slowly added in the necessary amount to reach the desired conversion. The reaction was left to proceed for 48 h and the esterified polymer was recovered by precipitation in diethyl ether, washed with acetone, and dried in vacuum for storage.

Esterification of PMLA with allyl alcohol was performed through activation of the carboxyl side group with DCC. Briefly, a mixture of 0.5 or 0.75 mmol of DCC in 2 mL of allyl alcohol was added dropwise to a solution of 1 mmol of PMLA in 2 mL of allyl alcohol; the reaction was left to proceed for 3 h at room temperature under magnetic stirring. The final reaction mixture was subjected to dialysis against methanol for 48 h using an 8 kDa cut-off membrane. The allyl ester of PMLA was lyophilized for recovery and storage.

8.2.2. Thiol-ene click reactions

The 3-alkylthio-propyl PGGA and PMLA esters were prepared as follows: To a 7.5 % (w/v) solution in NMP, at room temperature, of the allyl PGGA ester the corresponding 1-alkanothiol (1-octanethiol, 1-dodecanethiol and 1-hexadecanethiol) was added in a 2:1 molar ratio respect to double bond concentration, and then DMPA (4% respect to double bond) was added. The reaction mixture was placed under UV radiation (2 x Philips PL-S 11 W/10, 360 nm) and was irradiated for 24 h to reaction completion, reaction was followed by ^{1}H NMR; the grafted PGGA was recovered by pouring the reaction mixture into ethanol. The final product was dried in vacuum for storage. The same procedure was applied for the preparation of the PMLA derivatives but starting from a 1.5 % (w/v) of the allyl PMLA ester in DMSO.

8.3. Results and discussion

8.3.1. Comb-like polymers synthesis

Comb-like copolymers from PGGA and PMLA were obtained by a two-step process (Scheme 8.1). First, polymers were allylated in different degrees using specific procedures for PGGA and PMLA according to the

different susceptibility that their main chains display towards hydrolysis. PGGA was esterified with allyl bromide under middle-basic conditions whereas allyl alcohol was used for esterifying PMLA with the concourse of DCC as carboxylic side groups activator. Esterification was achieved with good yields, around 90 % for PGGA and above 75 % for PMLA derivatives (Table 8.1).

The resulting allylated copolymers were spectroscopycally pure as it was proved by ^{1}H NMR. In fact the occurrence of the allylation of the polyacids was clearly evidenced by following the changes taking place in the ^{1}H NMR spectra. For PGGA the signal of the allylic CH_2 appearing at 4.1 ppm in allyl bromide (Figure 8.1b, signal *e*) shifted 0.5 ppm downfield in *co*PGGA-Al$_x$H$_y$ (Figure 8.1c, signal *e*'). In the case of PMLA, a similar displacement was observed for the allylic CH_2 signal which moved 0.7 ppm downfield when the allyl alcohol entered in the PMLA as alcohyl group (signal *e* in Figure 8.2b compared to signal *e'* in Figure 8.2c). Molecular weight increases proportional to the modification degree, while copolymers polydispersities had similar values (Table 8.1).

Grafting of three linear alkanothiols containing 8 (Oc), 12 (doD) and 16 (hxD) carbons length, on allyl functionalized polymers, was successfully achieved taking advantage of the high reactivity and specificity of the thiol-ene *click* reaction. DMPA was chosen as photoiniciator and UV radiation as activator; the reaction is known to take place as a free radical reaction initiated by decomposition of DMPA and to proceed with propagation to terminate by radical chain transfer.[180] Conversion degrees for all copolymers was practically 100%

Scheme 8.1. Two-step grafting of aliphatic long chains into: a) PGGA and b) PMLA.

Table 8.1. Step 1: reaction conditions, conversion degrees, yields and molecular weights obtained for the different polymers.

	Feed[a]	Esterification %	Yield %	M_w[b]	D[b]
PGGA	-	-	-	30,000	
*co*PGGA-$Al_{25}H_{75}$	1 : 0.25	26	88	30,600	2.9
*co*PGGA-$Al_{50}H_{50}$	1 : 0.50	55	96	37,900	2.3
*co*PGGA-$Al_{75}H_{25}$	1 : 0.75	73	92	44,200	2.6
PMLA	-	-	-	25,000	1.2
*co*PMLA-$Al_{50}H_{50}$	1 : 0.50	48	79	27,300	2.8
*co*PMLA-$Al_{75}H_{25}$	1 : 0.75	73	75	33,500	3.0

[a] Molar ratio of: a) polymer:allyl bromide for PGGA; and b) polymer:DCC for PMLA reactions.
[b] Weight-average molecular weight and dispersity estimated by GPC.

as it could be assessed by ^{1}H NMR which showed that signals arising from the double bound (5-6 ppm) had fully disappeared after reaction (Figure 8.1d and 8.2d), indicating that the totality of allyl groups hadbeen converted. Nevertheless, reactions yields were relatively lower than for the first step, i.e. between 60 and 80 % for PGGA and around 45 % for PMLA derivatives (Table 8.2). Material looses could be due in part to photodegradation taking place by the prolonged exposition of the polymer to UV radiation. Shorter exposition times were assayed but reaction did not reach completeness. This degradation is also reflected in the molecular weight of the grafted copolymers which were found to be slightly lower than for their respective allylated precursors. Nevertheless, M_w values obtained for the three alkylated derivatives for each copolymer composition were consistent with the length of the alkylthiol chain length grafted in each case (Table 8.2).

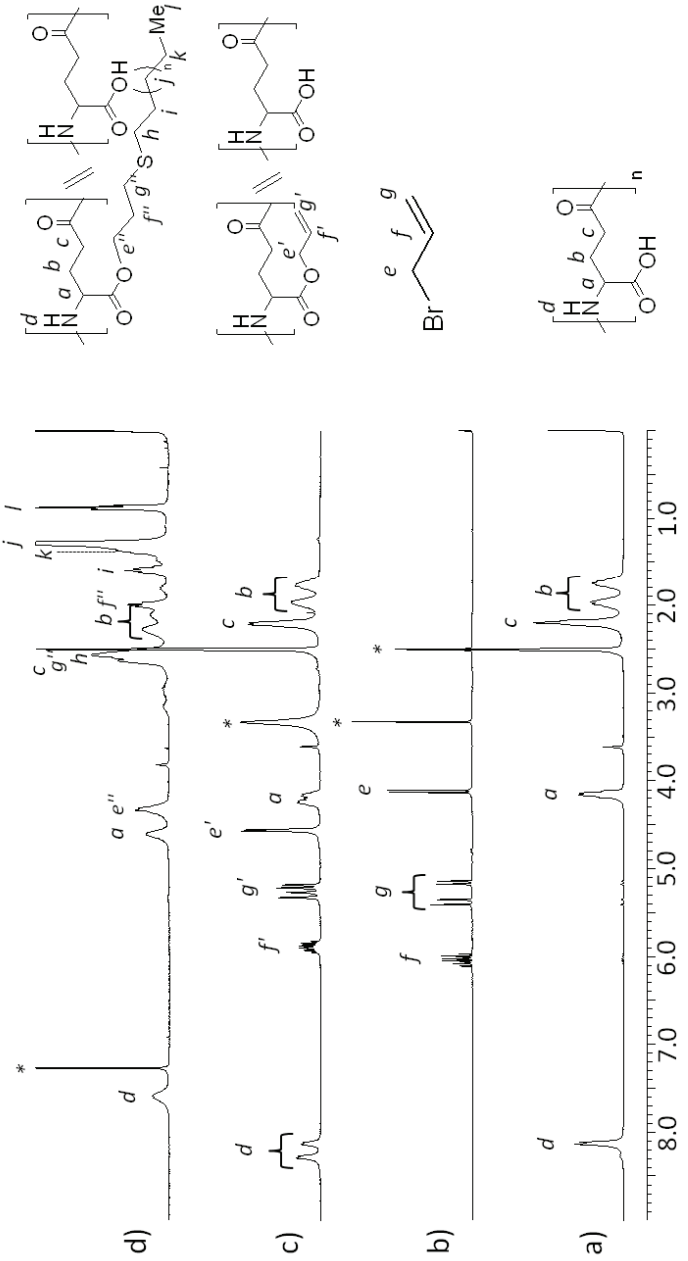


Figure 8.1. ^{1}H NMR spectra of: a) PGGA, b) allyl bromide, c) *co*PGGA-Al$_x$H$_y$ and d) *co*PGGA-PrSOc$_{50}$H$_{50}$. *Solvent.

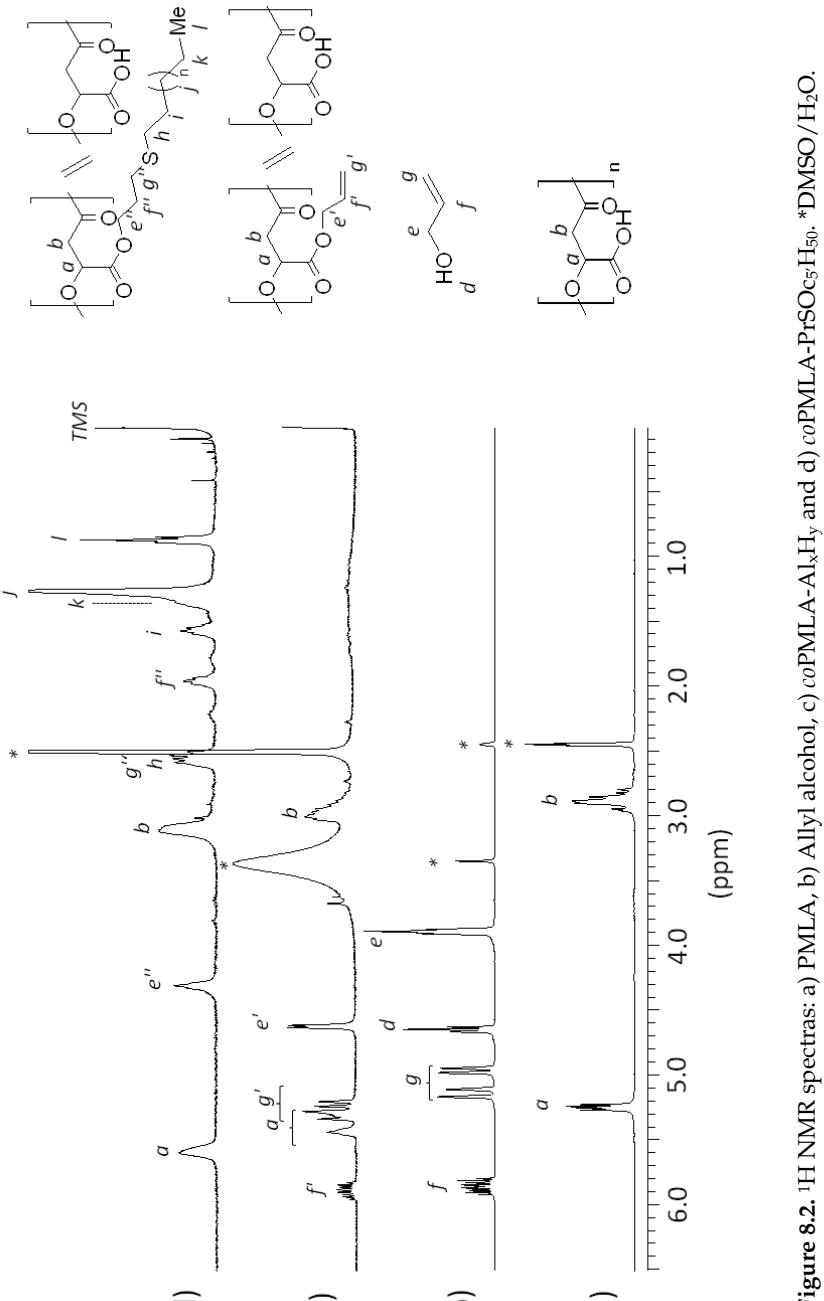


Figure 8.2. ^{1}H NMR spectras: a) PMLA, b) Allyl alcohol, c) *co*PMLA-Al_XH_y and d) *co*PMLA-PrSOc_5H_{50}. *DMSO/H_2O.

Table 8.2. Step 2: Results of the thiol-ene *click* reaction on allylated PGGA and PMLA.

Polymer	Conversion %	Yield %	M_w	Pd.
*co*PGGA-PrSO$c_{25}H_{75}$	100	61	25,200	2.5
*co*PGGA-PrSdo$D_{25}H_{75}$	100	62	27,800	2.6
*co*PGGA-PrShx$D_{25}H_{75}$	100	78	30,600	2.8
*co*PGGA-PrSO$c_{50}H_{50}$	100	67	29,000	2.9
*co*PGGA-PrSdo$D_{50}H_{50}$	100	74	31,800	2.3
*co*PGGA-PrShx$D_{50}H_{50}$	100	64	33,200	2.3
*co*PGGA-PrSO$c_{75}H_{25}$	100	60	30,300	2.6
*co*PGGA-PrSdo$D_{75}H_{25}$	100	79	33,700	2.8
*co*PGGA-PrShx$D_{75}H_{25}$	100	76	35,300	3.0
*co*PMLA-PrSO$c_{50}H_{50}$	100	49	31,000	2.1
*co*PMLA-PrSdo$D_{50}H_{50}$	100	49	32,100	2.2
*co*PMLA-PrShx$D_{50}H_{50}$	100	50	33,400	2.4
*co*PMLA-PrSO$c_{75}H_{25}$	100	51	33,100	2.0
*co*PMLA-PrSdo$D_{75}H_{25}$	100	47	36,300	2.4
*co*PMLA-PrShx$D_{75}H_{25}$	100	56	38,700	2.8

8.3.2. Thermal characterization

A DSC calorimetric study was carried out in order to appraise the crystalline character of the grafted copolymers since previous works on comb-like polyacids had shown that in these polymers long linear alkyl side chains are able to crystallize in a phase separated from the another one constituted by the main chain.[77,181,182] As it is observed in Figures 8.3 and 8.4, only copolymers bearing -SR side chains with 16 carbon atoms display endothermal peaks characteristic of melting, no matter what copolymer is concerned or what is the attained esterification degree.Melting temperatures are near to 55 °C for all the semicrystalline

polymers here examined, which is much expected value for the melting of a crystalline paraffinic phase made of the alkyl chains of sixteen carbon atoms. These results are consistent with what has been reported for poly(α-alkyl-γ-glutamate)s which were found to be able to crystallize for alkyl chains containing at least 14 carbon atoms.[77] It can be concluded therefore from these results that the sulphur atom is unable to enter in the paraffinic crystal lattice and therefore the propylthio group is rejected from the crystallized paraffinic phase to remain in a disordered interphase connecting the crystallized side chains and the polypeptide or polyester main chains.

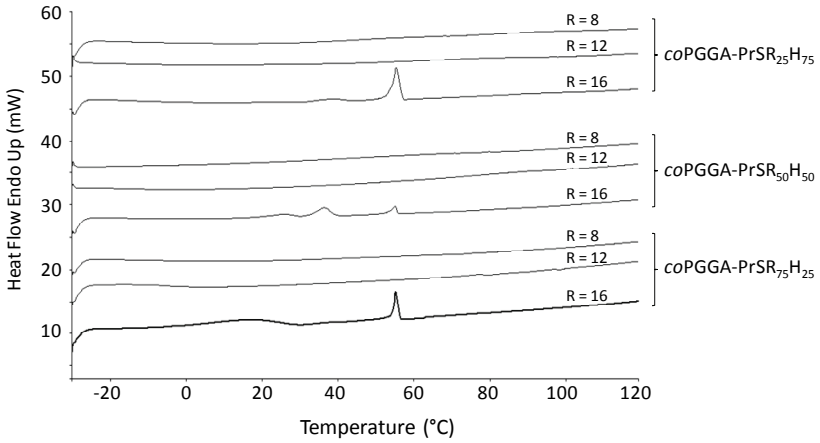


Figure 8.3. DSC profiles (first heating) of *co*PGGA-PrSR$_x$H$_y$.

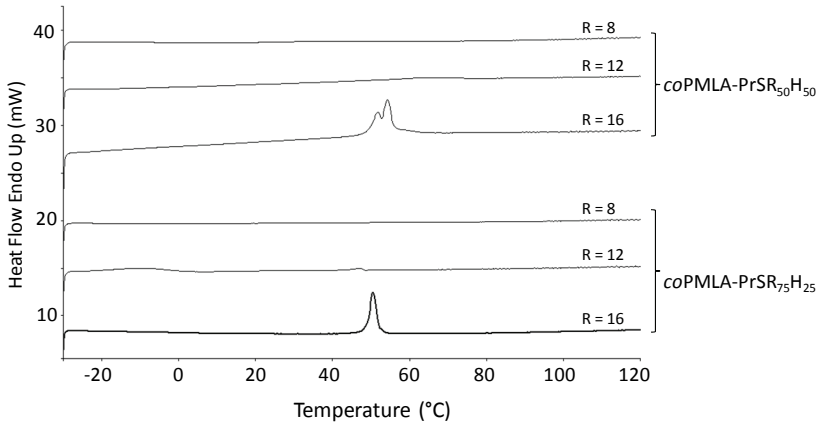


Figure 8.4. DSC profiles (first heating) of *co*PMLA-PrSR$_x$H$_y$.

8.3.3. Nanoparticles formation and characterization

Partial modification of the hydrophilic polyacids by alkylation resulted in amphiphilic macromolecules capable to form self-assembled nanostructures. To take advantage of this character, for nanoparticle formation the precipitation dialysis method was applied to modified polyacids. More or less spherical nanoparticles were obtained for *co*PGGA-PrSR$_{50}$H$_{50}$, *co*PGGA-PrSR$_{75}$H$_{25}$, *co*PMLA-PrSR$_{50}$H$_{50}$ and *co*PMLA-PrSR$_{75}$H$_{25}$ (Figure 8.5 and 8.6), whereas *co*PGGA-PrSR$_{25}$H$_{75}$ were not capable to form nanoparticles under the tested conditions. Average hydrodynamical diameters of these particles oscillated from 80 to 240 nm (Table 8.3), with smaller sizes obtained for PGGA derivatives. PGGA with 50% of free carboxylic groups, *co*PGGA-PrSR$_{50}$H$_{50}$, rendered smaller particles for longer grafted aliphatic chains. In the case of the PMLA series, larger particles were those prepared from derivative bearing side alkyl chains of intermediate length. What it is common to all the series is that smallest particle sizes are invariably obtained for compounds in which the alkyl chains are crystallized if exception is made for the *co*PGGA-PrShxD$_{75}$H$_{25}$ where very close particle diameter were obtained for the three alkyl chain lengths.

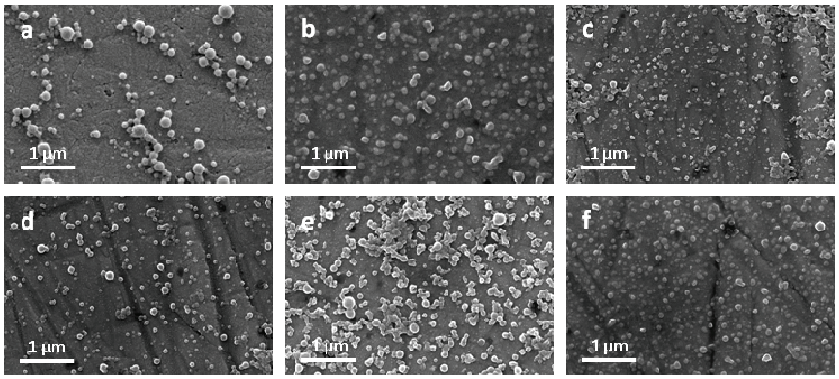


Figure 8.5. SEM micrographs of modified PGGA nanoparticles: a) *co*PGGA-PrSOc$_{50}$H$_{50}$, b) *co*PGGA-PrSdoD$_{50}$H$_{50}$, c) *co*PGGA-PrShxD$_{50}$H$_{50}$, d) *co*PGGA-PrSOc$_{75}$H$_{25}$, e) *co*PGGA-PrSdoD$_{75}$H$_{25}$ and f) *co*PGGA-PrShxD$_{75}$H$_{25}$.

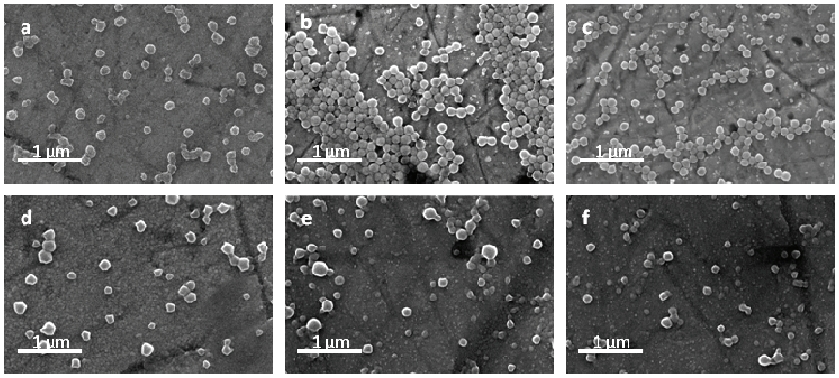


Figure 8.6. SEM micrographs of modified PMLA nanoparticles: a) *co*PMLA-PrSOc$_{50}$H$_{50}$, b) *co*PMLA-PrSdoD$_{50}$H$_{50}$, c) *co*PMLA-PrShxD$_{50}$H$_{50}$, d) *co*PMLA-PrSOc$_{75}$H$_{25}$, e) *co*PMLA-PrSdoD$_{75}$H$_{25}$ and f) *co*PMLA-PrShxD$_{75}$H$_{25}$.

Particle surface charge was estimated as the ζ-potential measured in distilled water; results are included in Table 8.3. As it was expected, all nanoparticles presented negative values due to the remaining free carboxylic units still present in all compounds from both PGGA and PMLA. On the basis of well-settled amphiphilic self-assembled particle

Table 8.3. Nanoparticles characterization of the different copolymers. Drug content and encapsulation efficiency are presented for Carbamazepine and Theophylline.

	Size (nm)	Pd.I.[a]	ζ-pot (mV)	CBZ Cont. (%)	CBZ E.E. (%)	TEO Cont. (%)	TEO EE (%)
*co*PGGA-PrSO$c_{50}H_{50}$	194	0.408	-30.2	3.18	15.9	0.54	2.7
*co*PGGA-PrSdo$D_{50}H_{50}$	157	0.294	-33.5	4.15	20.7	0.65	3.2
*co*PGGA-PrShx$D_{50}H_{50}$	76	0.155	-32.1	7.68	38.4	1.74	8.7
*co*PGGA-PrSO$c_{75}H_{25}$	138	0.157	-29.9	5.36	26.8	2.70	13.5
*co*PGGA-PrSdo$D_{75}H_{25}$	135	0.160	-28.4	3.38	16.9	1.99	9.9
*co*PGGA-PrShx$D_{75}H_{25}$	144	0.203	-28.5	1.85	9.2	1.61	8.0
*co*PMLA-PrSO$c_{50}H_{50}$	197	0.383	-40.6	5.8	29.0	4.1	20.5
*co*PMLA-PrSdo$D_{50}H_{50}$	236	0.544	-32.3	6.4	32.0	3.8	19.0
*co*PMLA-PrShx$D_{50}H_{50}$	170	0.335	-29.0	6.9	34.5	2.8	13.9
*co*PMLA-PrSO$c_{75}H_{25}$	206	0.343	-35.3	5.9	29.5	1.7	8.5
*co*PMLA-PrSdo$D_{75}H_{25}$	239	0.486	-24.8	5.7	28.5	1.1	5.5
*co*PMLA-PrShx$D_{75}H_{25}$	151	0.348	-12.7	4.9	24.5	1.4	7.0

[a] Dispersity index of particle sizes.

structure concept it can be reasonably assumed that the carboxylic grafted chains are hidden in the inner part to form a core-shell structure. The trend observed for the ζ-potential values along the series and even within each series deserves comments. In first place it must be remarked that higher values and differences observed for the PMLA derivatives can be due to the greater ability of the more flexible polymalate chain to be sterically accommodated in the particle as a response to environment interactions. When different conversions are compared the negative charge decreases with increasing conversion degree, which is in accordance with what should be expected for the variation in the negative hydrophilic/neutral hydrophobic ratio. Lastly it is also remarkable that in PMLA derivatives the minimum ζ-potential values correspond to compositions in which the alkyl side chains are crystallized.

8.3.4. Hydrolytic degradation

The hydrolytic degradation of the nanoparticles was examined upon incubation under physiological conditions, pH 7.4 and 37 °C (Figure 8.7). Degradation rate was determined by following the molecular weight reduction as a function of incubation time. As it was expected the susceptibility of nanoparticles to hydrolysis decreased for higher conversion degrees and longer alkyl side chain lengths. Both factors, modification degree and lateral chain length, are directly related to the material hydrophobicity, and therefore with the sensitiveness to hydrolysis of the copolymers. PMLA copolymer derivatives displayed a much higher rate of hydrolysis than those of PGGA because the higher lability of the main chain ester bond compared to the amide bond of PGGA. The degradation profiles depicted in Figure 8.7 show that after 12 weeks of incubation *co*PGGA-PrSOc$_{50}$H$_{50}$ retained about 60 % of its original molecular weight, while *co*PGGA-PrShxD$_{50}$H$_{50}$ remained above 80 %. Conversely, in PMLA derivatives, the *co*PMLA-PrSOc$_{50}$H$_{50}$

molecular weight fell down to 35 % of its original value while for *co*PMLA-PrShxD$_{50}$H$_{50}$ it remained above 70 %. Polymers with 75 % of their units modified, hydrolytic degradation is slight, only *co*PGGA-PrSOc$_{75}$H$_{25}$ and *co*PMLA-PrSOc$_{75}$H$_{25}$ showed a considerable molecular weight reduction, nevertheless those with longer side chains left over more than 90 % of the original molecular weight.

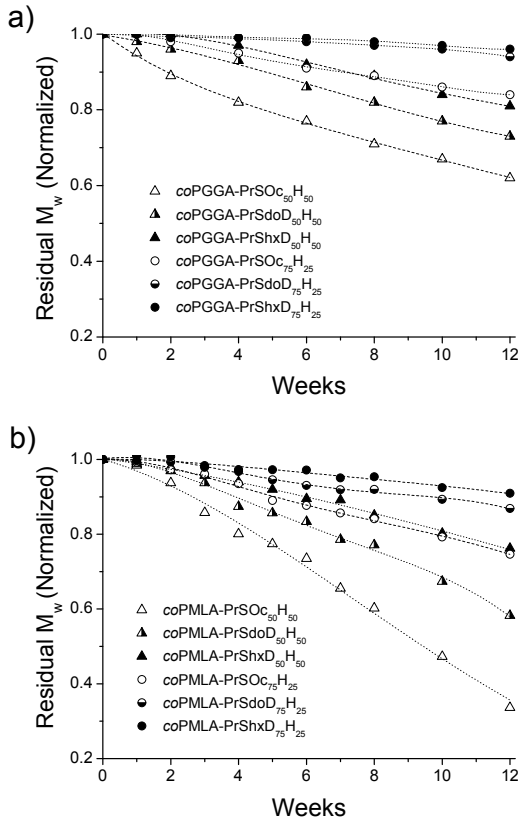


Figure 8.7. Evolution of the molecular weight of copolymers incubated under physiological conditions a) *co*PGGA-PrSR$_x$H$_y$ and b) *co*PMLA-PrSR$_x$H$_y$.

Hydrolytic degradation mechanism was studied with the support of ^{1}H NMR analysis of the soluble products released to the aqueous medium upon incubation. According to the results found in the

degradation rate assays, copolymers modified at lower conversion degrees with shorter alkyl side chains degraded considerably faster and PGGA series were more resistant than the PMLA ones. Nevertheless the degradation mechanism seems to be common to all of them. Since *co*PGGA-PrSO$c_{50}H_{50}$ and *co*PMLA-PrSO$c_{50}H_{50}$ are the most degraded samples and their degradation products are water-soluble, they were chosen to describe the degradation mechanism operating in the PGGA and PMLA copolyesters series, respectively.

In the degradation of *co*PGGA-PrSO$c_{50}H_{50}$, first observable signals appeared after one month of incubation. They were assigned to protons attached to the main chain carbons of glutamyl residues and to the protons contained in the alkylthioalkanol chains indicating the hydrolysis of the side ester group with the subsequent solubilization of fragments enriched in free carboxylic groups. In the second month of degradation, signals from the polymer terminal groups became observable, revealing that hydrolysis of the amide bonds from the main chain has started. From the third month ahead, signals got intensified, and those from the main chain displayed much better resolution. The progressive degradation of the main chain was evidenced by a clear increase of terminal groups signals. The occurrence of free glutamic acid in the aqueous medium could not be ascertained because its signals, if present, overlapped with those arising from both polymer and oligomer species (Figure 8.8).

For PMLA derivatives the mechanism was found to be similar although degradation happened in shorter time. In this case, first signals appearing just after one week of incubation corresponded to the protons of free malic acid together with those arising from the hydrolyzed lateral chain, indicating that hydrolysis of ester groups, both the main chain and the side chain, took place at the same time. Along the two first months of incubation no signals corresponding to terminal groups or to main chain methylene units were observed, this confirms that until the side chain hydrolysis occur, the copolymers are insoluble, then the fast

monomer PMLA cleavage occurs. Spectras became simplified and intensified with time so at the end of the incubation period, the only visible signals were those arising from free malic acid and the alkylthioalkanol (Figure 8.9).

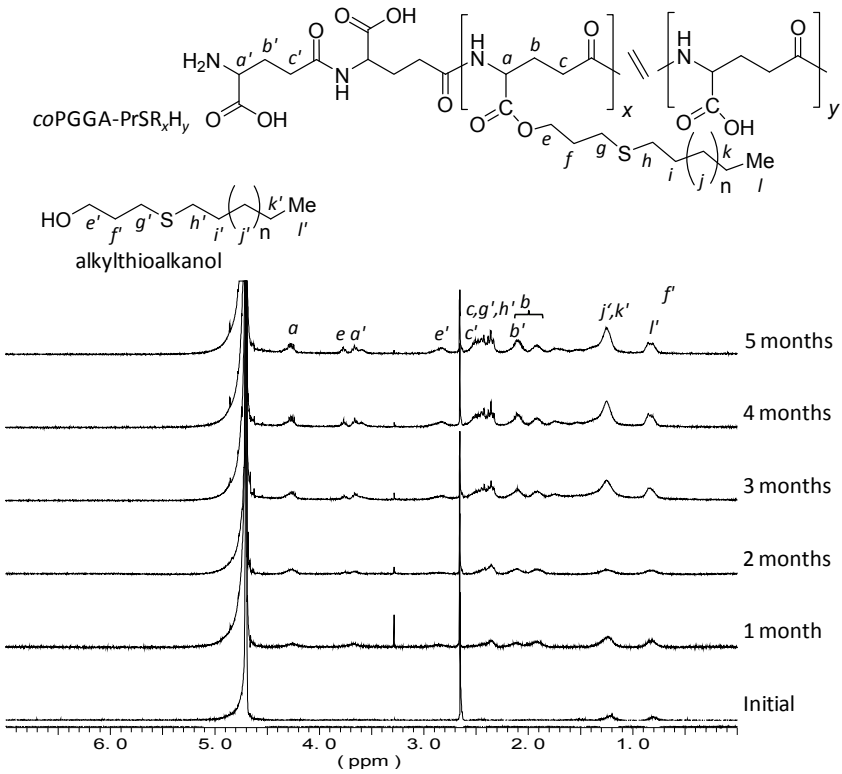


Figure 8.8. Evolution of the ^{1}H NMR spectrum taking from the aqueous medium of incubated *co*PGGA-PrSOc$_{50}$H$_{50}$ nanoparticles along time.

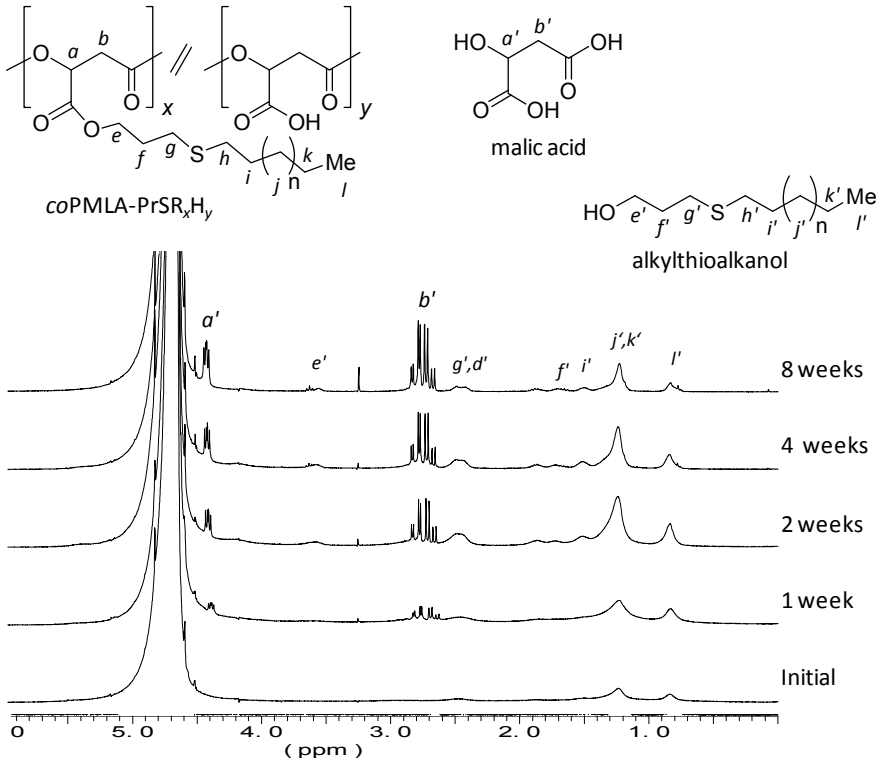


Figure 8.9. Evolution of the ^{1}H NMR spectrum taking from the aqueous medium of incubated *co*PMLA-PrSO$c_{50}H_{50}$ nanoparticles along time.

8.3.5. Drug encapsulation and in vitro release

Encapsulated compounds were Carbamazepine (CBZ) and Theophylline (TEO); both of them used as models for hydrophobic and hydrophilic drugs, respectively, for encapsulation and releasing studies. The encapsulation method applied in this work made use of the precipitation dialysis method used for nanoparticle formation. The drug was added to the polymer solution prior to nanoparticle formation. Results are summarized in Table 8.3. CBZ appeared to be able to be encapsulated better than TEO displaying maximum encapsulation efficiency (EE) in *co*PMLA-PrShxD$_{50}H_{50}$ nanoparticles with 34 % of EE compared to only 20 % for TEO, while TEO only reaches 20 % with the

most hydrophilic polymer of all, *co*PMLA-PrSOc$_{50}$H$_{50}$. The low encapsulation efficiency of TEO may be because, during dialysis, drug losses are greater than in the case of CBZ due to their hydrophilicity.

In vitro drug release assays were carried out under physiological conditions, pH 7.4 and 37 °C. Both drugs presented a burst release within the first hours of incubation, a fact that was accentuated for encapsulated TEO nanoparticles, which released between 60 and 80 % of the loaded drug in the first two hours of incubation. On the contrary, CBZ loaded nanoparticles showed a more controlled release, with a 40 to 60 % release of the loaded drug after 6 hours of incubation (Figure 8.10). Since nanoparticles of the same polymer are being compared it can be inferred that differences in drug release are largely determined by the hydrophobicity of the encapsulated compound. Given the obtained

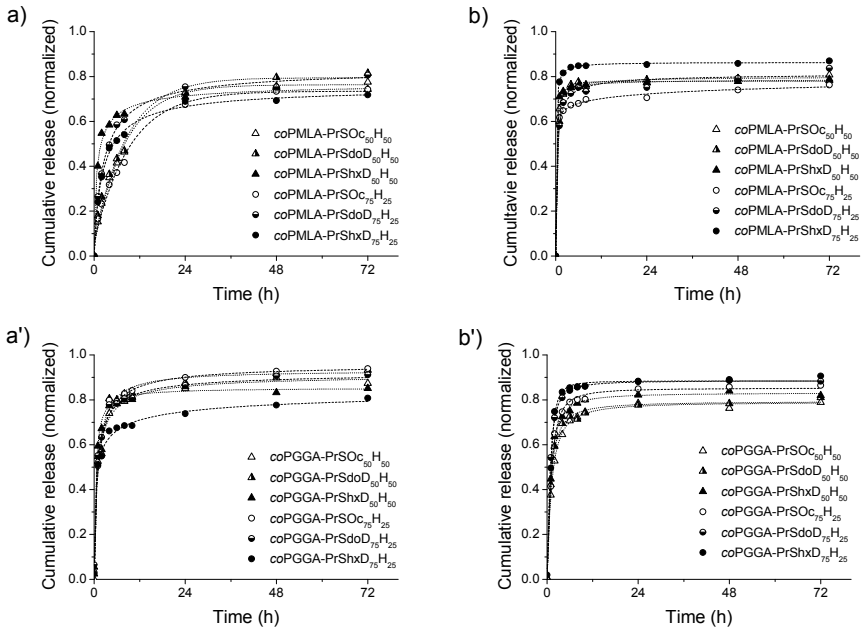


Figure 8.10. Drug release profiles for *co*PMLA-PrSR$_x$H$_y$ (top) and *co*PGGA-PrSR$_x$H$_y$ (bottom) nanoparticles incubated under physiological conditions and loaded with Carbamazepine (a, a') and Theophyline (b, b').

encapsulation efficiency for CBZ and the more sustained release of this drug, it can be concluded that the derivatives examined in this work as well as the applied encapsulation method are more suitable for the design of DDS systems loaded with hydrophobic drugs.

To understand the process that is behind the drug release from the nanoparticles CBZ loaded-films of both PGGA and PMLA copolymers were prepared and subjected to release assays. Films were analyzed by DSC and compared to the pure polymer and the physical blend to get insight of the state in which CBZ is in the film. The DSC trace of CBZ showed a characteristic melting peak around 180-190 °C, which it is also present in the traces from the physical blends but absent in the traces of the drug-loaded film, suggesting that the drug is well dispersed in the polymeric matrix (Figure 8.11). Although the release of the drug from the film took place at slower rates than for nanoparticles it was almost completely liberated in the first two days (Figure 8.12). It can be concluded therefore that drug release of CBZ from these materials is governed by a diffusion process since the degradation of the copolymers take place in much longer periods of time. Release rates were found to be faster for films made of copolymers with lower modification degrees and those modified with shorter aliphatic chains. Again drug release has to be related to the hydrophobicity of the material and the water penetration capability. Given the similitude of behavior with that observed for nanoparticles, the same diffusion process must govern drug release in both systems.

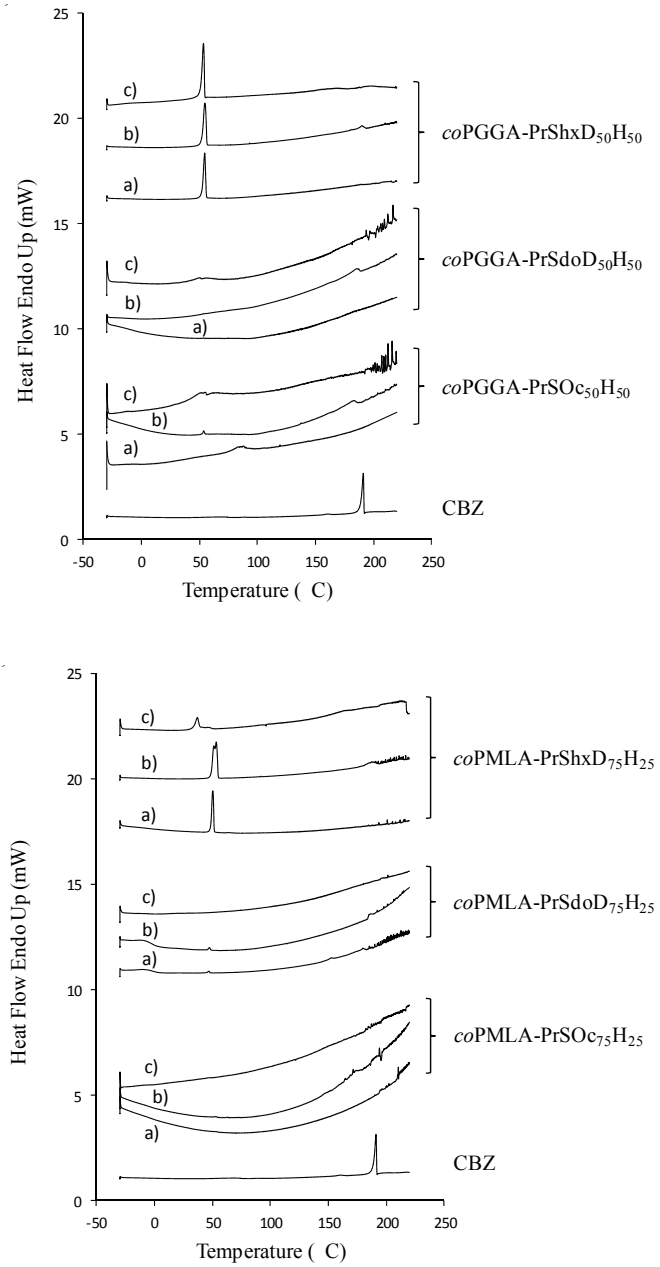


Figure 8.11. DSC traces of CBZ and a) polymer, b) physical blend polymer + CBZ and c) CBZ -loaded film. Top: *co*PGGA-PrSR_xH_y and bottom: *co*PMLA-PrSR_xH_y.

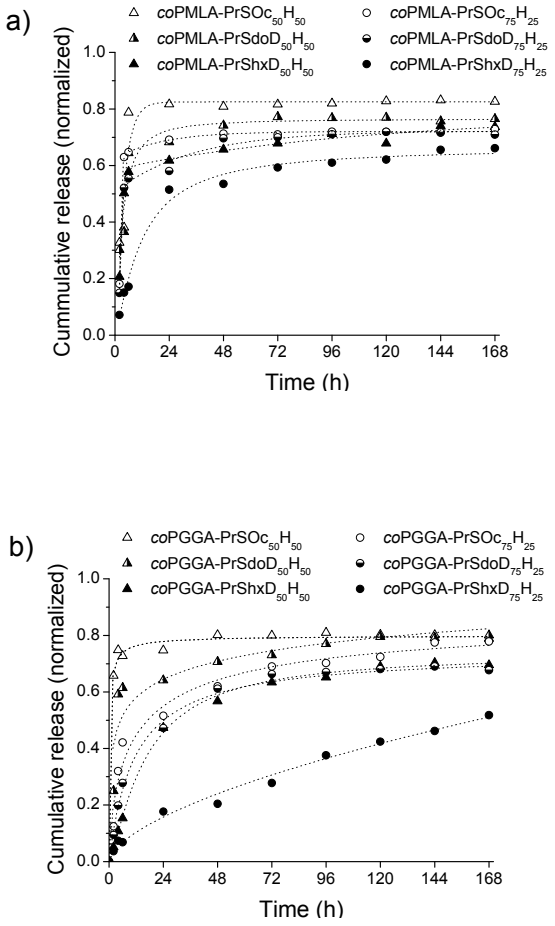


Figure 8.12. Carbamazapine release from drug-loaded films. a) *co*PMLA-PrSR$_x$H$_y$ and b) *co*PGGA-PrSR$_x$H$_y$.

8.4. Conclusions

Microbial polymalic and polyglutamic acids were modified by grafting aliphatic long chains through a two step process that makes use of thiol-ene *click* reactions. The prepared biodegradable polymers presented an amphiphilic character which drives their self-assembling in aqueous media into nanostructurated particles capable of drug encapsulation and suitable as drug delivery systems. Nanoparticles varied in size from 80 to 240 nm, the smaller ones being those prepared from PGGA derivatives. Hydrolytic degradation was found to be faster for PMLA than for PGGA derivatives as correspond to expected differences between polyesters and polyamides. Degradation happened by releasing the corresponding thioether alcohol and either malic or glutamic acid to the medium upon degradation. Model drugs TEO and CBZ were encapsulated in polymer nanoparticles with better efficiency for the hydrophobic drug CBZ than for the hydrophilic TEO. Both drugs were released upon incubation at pH 7.4 and 37 ˚C; with a burst release taking place in the first few hours and with almost complete release in 24 h. The CBZ release profile obtained from drug-loaded films suggested that the drug is liberated through a mediated-diffusion process.

9

General Conclusions

i. The biotechnological polymer poly(β,L-malic acid) (PMLA) was successfully modified, by direct esterification or amidation of its pendant carboxylic groups, under soft conditions without significant degradation. At the same time, PMLA and poly(γ-glutamic acid) (PGGA) derivatization through *click* reaction was possible, opening a new window for orthogonal modification of PMLA and PGGA. Polymer modification led to a series of derivatives which showed significant changes in the original physicochemical properties.

ii. PMLA esterification and amidation with organic compounds resulted in hydrophobic or amphiphilic polymers, depending on the attained conversion, which were capable of forming nanoparticles suitable for drug encapsulation and controlled release, either by emulsion solvent evaporation or precipitation dialysis techniques. Nanoparticles size varied between 70 to 350 nm, smaller size being obtained for those prepared by precipitation dialysis.

iii. Hydrolytic degradation of all esterified derivatives happened by hydrolysis of the lateral ester group followed by the scission of the main chain hidrolizable bonds, with subsequent release to the degradation medium of the grafted molecule and easily metabolizable malic acid or glutamic acid, as unique final products. Degradation occurred in a time scale of weeks, at a rate

that depended on the modification degree and on the hydrophobic character of the grafted molecules. Hydrolytic degradation was found to be faster for PMLA than for PGGA derivatives as correspond to expected differences between polyesters and polyamides. Furthermore, the presence of emulsifier on particles surface acted as a hydrolysis protecting coat.

iv. Temozolomide (TMZ) and Doxorubicin (DOX) were encapsulated in modified PMLA by different methods. Better efficiency was obtained for emulsion solvent evaporation over precipitation dialysis technique. However, the ionic coupling of DOX with pristine PMLA showed to be a highly effective and simple method for DOX loading, since ionized PMLA interacted efficiently with DOX to form stable ionic complexes.

v. DOX and TMZ were encapsulated in PMLA derivatives nanoparticles and were released upon incubation under physiological conditions. TMZ encapsulation afforded protection to the drug against hydrolytic decomposition. Release of TMZ took place within a few hours with subsequent hydrolytic pH-dependent activation. DOX was released in a time scale of days, the delay thought to be caused by the intermolecular interactions of the drug with the unmodified carboxylic groups of the polymer as well as with other DOX molecules.

vi. PMLA and DOX formed stable complexes which tend to self-assemble in microparticles or nanoparticles according to their polymer/drug ratio. DOX release from these complexes resulted to be pH dependent, and in less degree, ionic strength reliant. This characteristic is of particular interest and can be used for site controlled drug delivery.

vii. Theophylline (THEO) and Carbamazepine (CBZ) were encapsulated in partially esterified PMLA and PGGA by precipitation dialysis method, with higher efficiency for the hydrophobic CBZ. The fast release from drug loaded nanoparticles and films made of long aliphatic chains PMLA and PGGA derivatives suggested the occurrence of a diffusion driven releasing process.

viii. PMLA methyl derivative resulted in some cytotoxicity at long time exposure to cells from released methanol resulting from polymer degradation. Cellular toxicity of this derivative was overcome by the use of ethyl, butyl and aminoacyl coupled PMLA. Amino acids derivatives did not show sign of toxicity during 24 hours of administration provided that the concentration of nanoparticles was kept below 0.125 $mg \cdot mL^{-1}$ in the culture medium.

ix. Drug-unloaded nanoparticles made of PMLA-Et and PMLA-Bu derivatives, were not cytotoxic for the tested cancer cell lines, whereas drug-loaded nanoparticles were toxic for the same cell lines. For MDA MB468 cells, TMZ loaded particles were highly efficient, while free TMZ did not show any relevant effect. Furthermore DOX loaded in nanoparticles made of copolymer *co*PMLA-$Et_{50}H_{50}$ showed better internalization by cells than free DOX.

x. New comb–like polymers obtained through functionalization of pendant carboxylic groups of PMLA and PGGA, were able to be mildly elaborated using thiol-ene UV-mediated *click* reaction; demonstrating the applicability of a versatile technique for orthogonal and multifunctional modification of microbial polymers for the design of biodegradable drug delivery systems.

xi. The relative easy modification procedures, the degradability of the derivatives obtained, their capability to form nanoparticles and to encapsulate drugs, the low toxicity to cells and good response of cells to drug-loaded nanoparticles make biotechnological PMLA derivatives attractive materials for the design and development of biodegradable drug delivery systems, in particular with potential for the therapy of some diseases considered today challenging to pharmacological treatment.

Appendix 1

Chapter 5 Support Information:

Nanoparticles of Esterified Polymalic Acid for Controlled Anticancer Drug Release

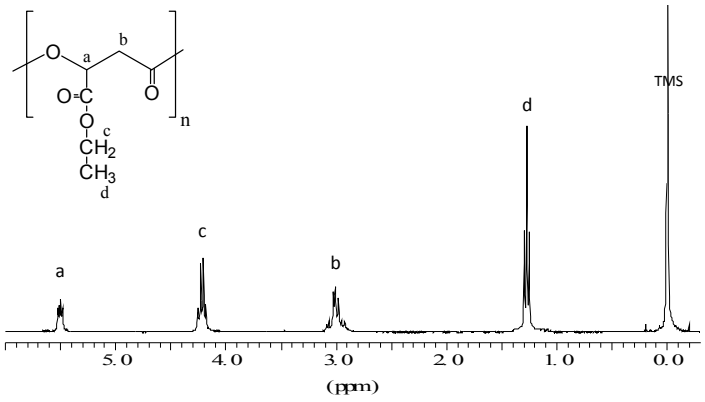


Figure A1.1. ^{1}H NMR spectrum of PMLA-Et_{100}.

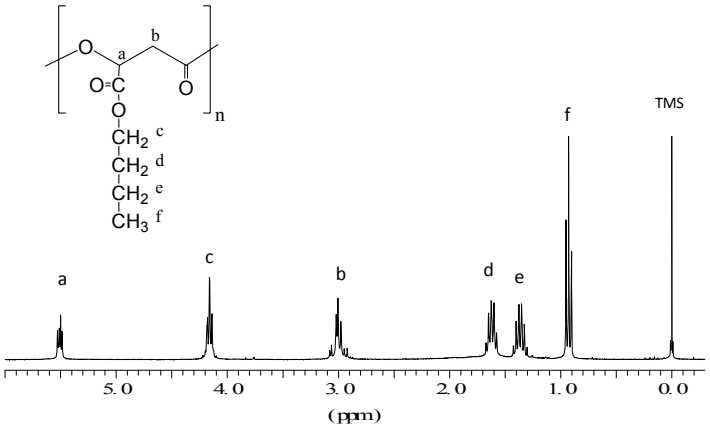


Figure A1.2. ^{1}H NMR spectrum of of PMLA-Bu_{100}.

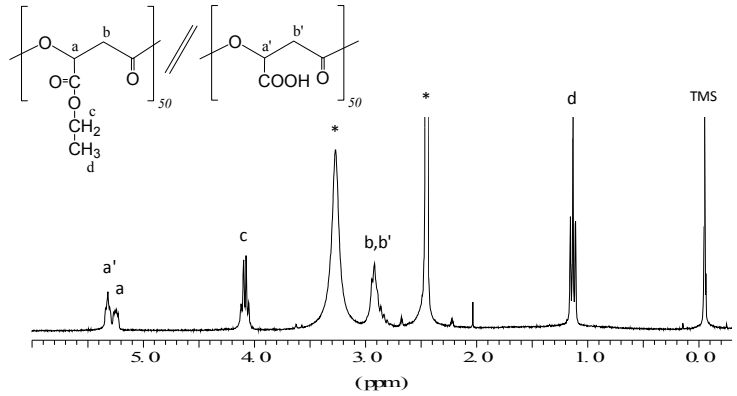


Figure A1.3. ^{1}H NMR spectrum of *co*PMLA-$Et_{50}H_{50}$. (*) DMSO and water solvent peaks.

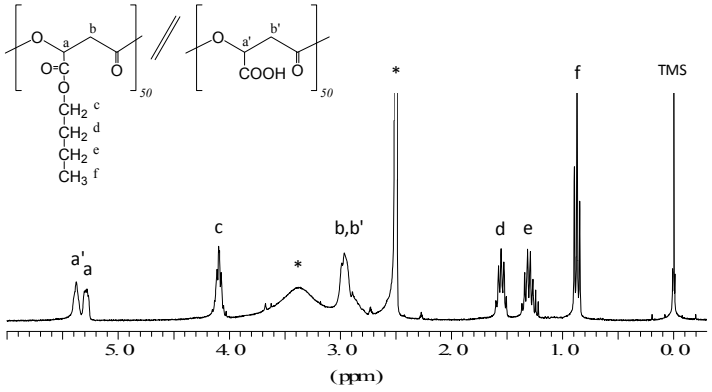


Figure A1.4. ^{1}H NMR spectrum of *co*PMLA-$Bu_{50}H_{50}$. (*) DMSO and water solvent peaks.

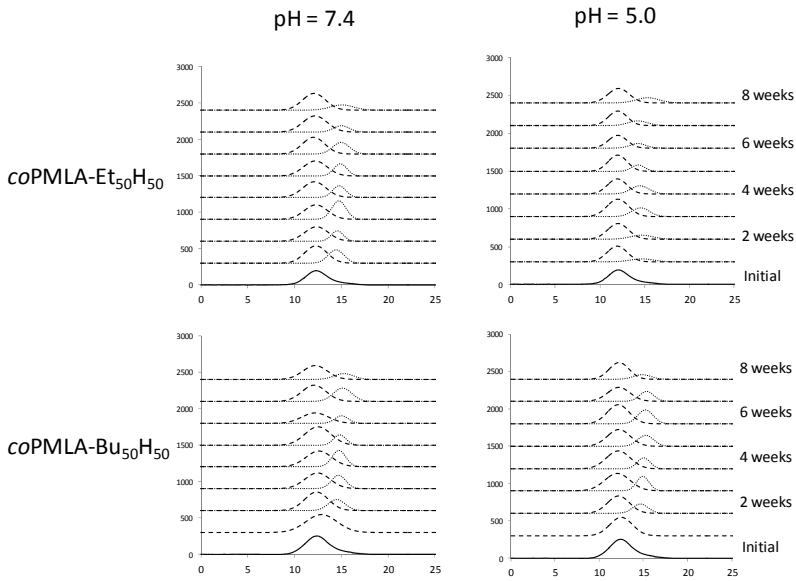


Figure A1.5. Deconvoluted GPC chromatograms of *co*PMLA-$Et_{50}H_{50}$ and *co*PMLA-$Bu_{50}H_{50}$ after incubation in aqueous buffer at pH 7.4 and 5.0 for the indicated times.

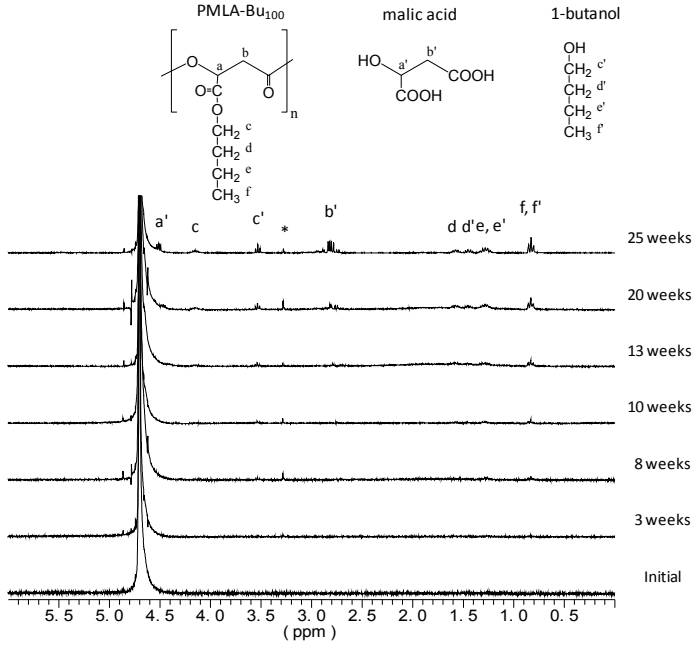


Figure A1.6. ^{1}H NMR spectra of the degradation products over time for PMLA-Bu_{100}. (*) impurity.

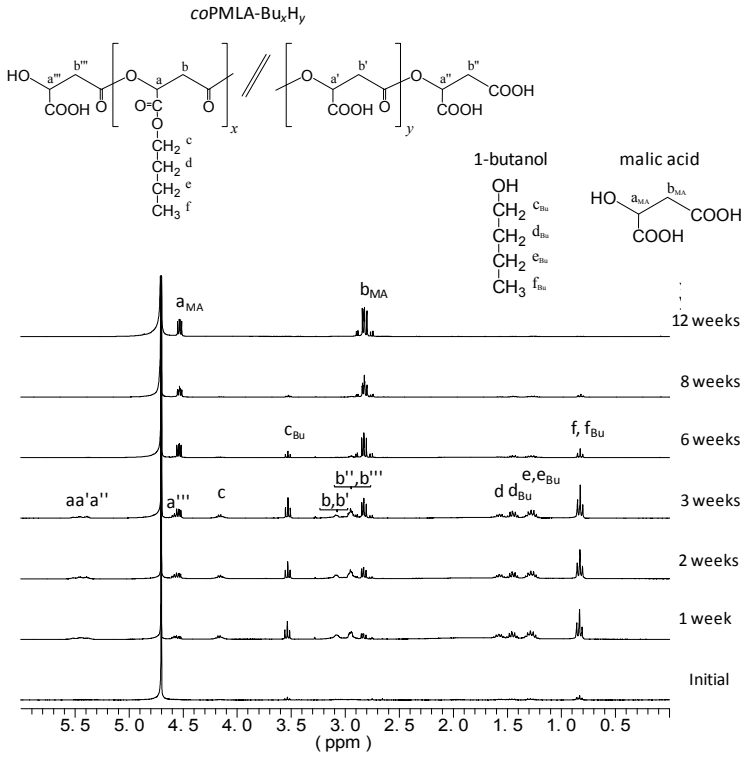


Figure A1.7. ^{1}H NMR spectra of the degradation products over time for *co*PMLA-$Bu_{50}H_{50}$.

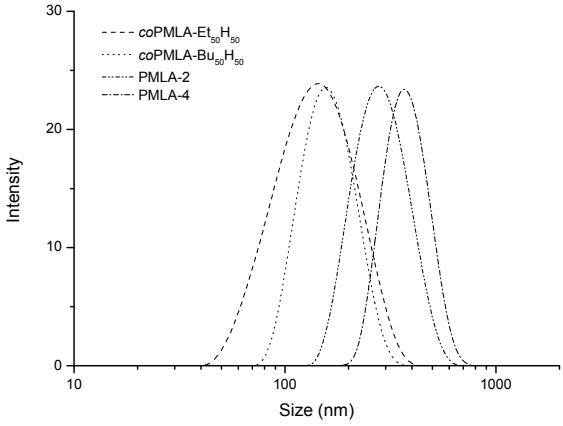


Figure A1.8. Particle size distributions measured by light scattering, distributions based on intensity of PMLA esterified derivatives.

Appendix 2

Chapter 7 Support Information:

Poly(β,L-Malic Acid)/Doxorubicin Ionic Complex: a pH-Dependent Delivery System

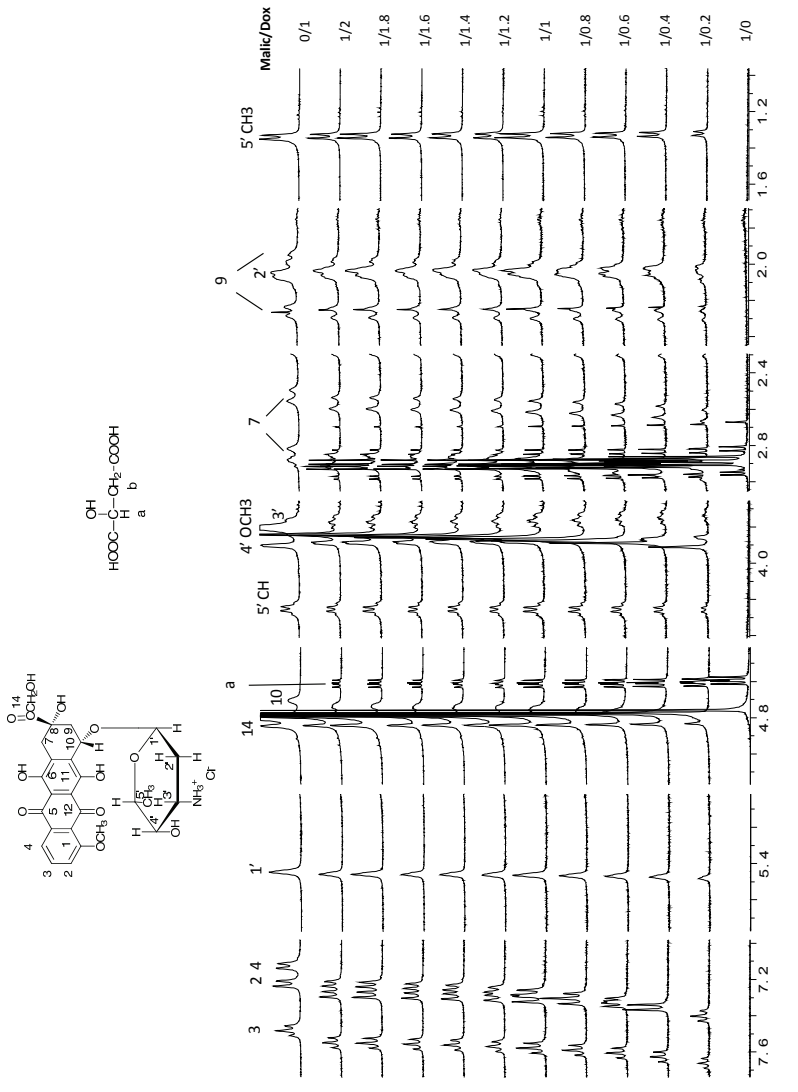


Figure A2.1. ^{1}H NMR spectra in D_2O of malic acid, DOX and the mixtures of the two compounds at the indicated molar ratios.

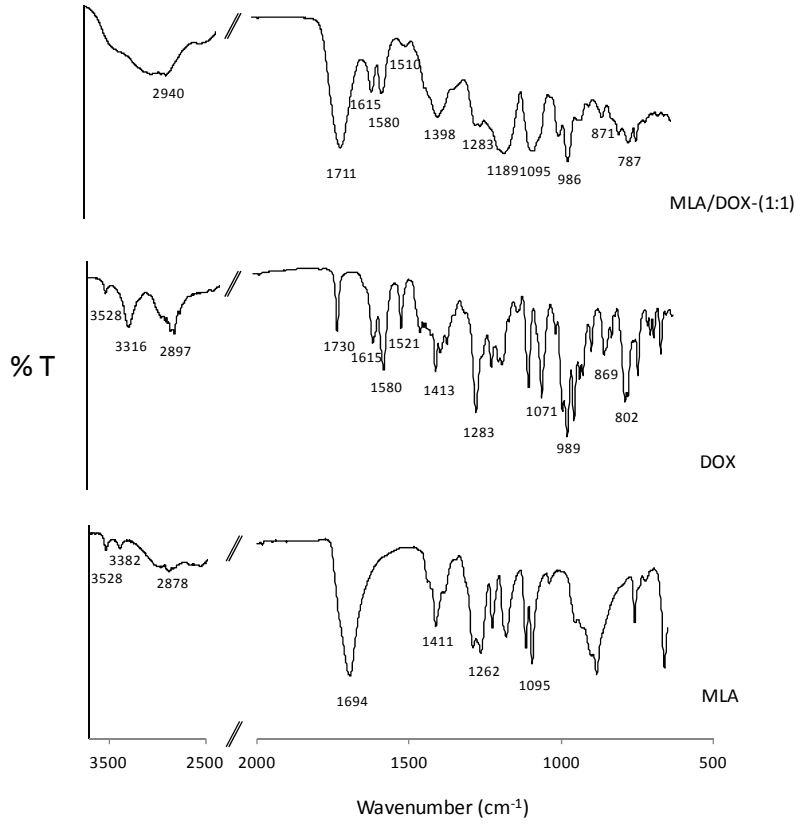


Figure A2.2. FTIR spectra of malic acid, DOX and the residue recovery from evaporation of the aqueous solution of an equimolar mixture of the two compounds.

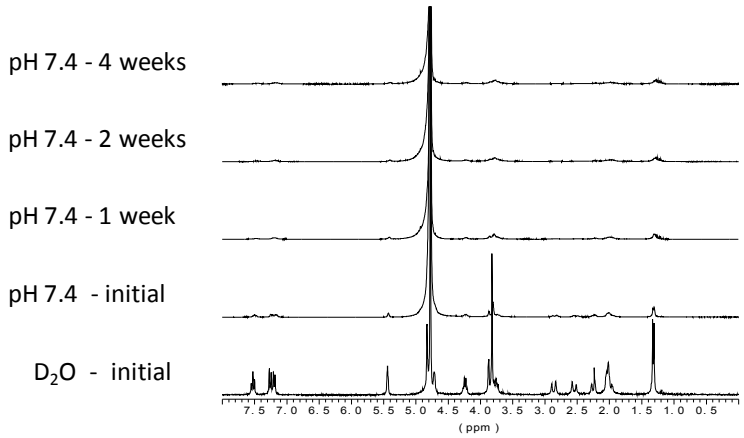


Figure A2.3. Evolution of ^{1}H NMR signals of DOX incubated in phosphate buffer pH 7.4 along time.

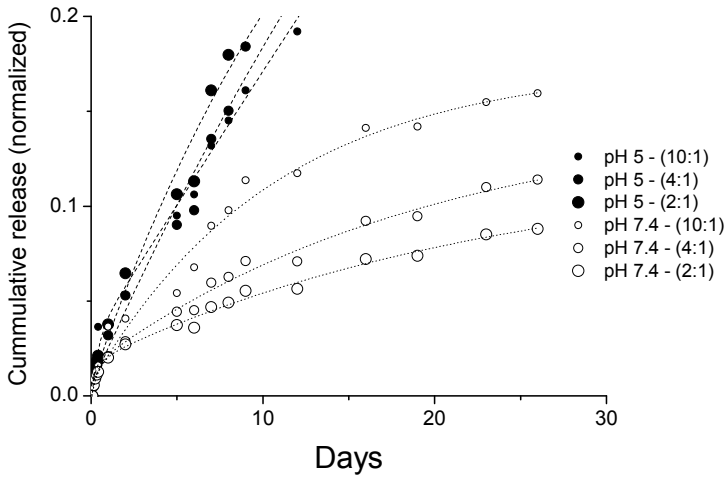


Figure A2.4. Figure 7.9a of the manuscript plotted at the same ordinate scale than Figure 7.9b to appraise the burst happening at the initial stage of the drug release from PMLA/DOX complexes.

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About the Author

Alberto Lanz Landázuri was born in Mexico City, the 16th of September of 1979. In 2001 he obtained his bachelor degree in Oceanography by the Autonomous University of Baja California (UABC). After some time working at a marine national park he got jaded of the plastic pollution of the seas. His urge and inquisitiveness took him to focus on biodegradable plastics. In 2006 he obtained a Biotechnology master degree, by the Northwest Biological Research Center (CIBNOR), with the screening and isolation of PHB producing marine bacteria. Still with the query on how to characterize, modify and apply biodegradable polymers he looked for a PhD. program in which could learn it. In 2008 he obtained a second master degree in Polymers and Biopolymers by the Polytechnic University of Catalonia (UPC) as beginning of his PhD. research work under the supervision of Dr. Sebastián Muñoz Guerra and Dra. Montserrat García Álvarez. The results generated in his research on modification of microbial polyacids for drug delivery systems are presented in this Thesis.

Publications

i. *Poly(Methyl Malate) Nanoparticles: Formation, Degradation, and Encapsulation of Anticancer Drugs.* A. Lanz-Landázuri, M. García-Alvarez, J. Portilla-Arias, A. Martínez de Ilarduya, R. Patil, E. Holler, J. Y. Ljubimova, S. Muñoz-Guerra, Macromol. Biosci., 2011, 11(10):1370-7.

ii. *Modification of Microbial Polymalic Acid with Hydrophobic Amino Acids for Drug-Releasing Nanoparticles.* A. Lanz-Landázuri, M. García-Alvarez, J. Portilla-Arias, A. Martínez de Ilarduya, E. Holler, J. Y. Ljubimova, S. Muñoz-Guerra, Macromol. Chem. Phys., 2012, 213:1623-1631.

iii. *Poly(β,L-Malic Acid)/Doxorubicin Ionic Complex: a pH-Dependent Delivery System.* A. Lanz-Landázuri, A. Martínez de Ilarduya, M. García-Alvarez, S. Muñoz-Guerra, React. Funct. Polym. (submitted).

iv. *Nanoparticles of Esterified Polymalic Acid for Controlled Anticancer Drug Release.* A. Lanz-Landázuri, J. Portilla-Arias, A. Martínez de Ilarduya, M. García-Alvarez, E. Holler, J. Y. Ljubimova, S. Muñoz-Guerra, Macromol. Biosci. (submitted).

v. *Modification of Biotechnological Polymers by Thiol-Ene Click Reaction: Nanoparticle Formation and Drug Encapsulation.* A. Lanz-Landázuri, A. Martínez de Ilarduya, M. García-Alvarez, S. Muñoz-Guerra, (in progress).

Communications

a) *Modificación del Acido Poli(β,L-Málico) con Aminoácidos Hidrofóbicos para la Formación de Nanopartículas.* A. Lanz Landázuri, M. García Álvarez, S. Muñoz Guerra. V Congress of Young Polymer Researchers, 2010, Spain.

b) *Amidation of Poly(β,L-Malic Acid) with Hydrophobic Amino Acids for Nanoparticles Formation and Drug Encapsulation* (poster). A. Lanz Landázuri, M. García Alvarez, S. Muñoz Guerra. 3rd International Conference on Biodegradable and Biobased Polymers, 2011, France.

c) *Poly(β,L-Malic Acid)/Doxorubicin Ionic Complex for Controlled Drug Delivery* (poster). A. Lanz-Landázuri, A. Martínez de Ilarduya, M. García-Alvarez, S. Muñoz-Guerra. Third International Symposium Frontiers in Polymer Science, 2013, Spain.

d) *Synthesis of Comb-Like Alkyl Polymalates Via Thiol-Ene Click Chemistry: Nanoparticles Formation and Drug Encapsulation* (poster). A. Lanz-Landázuri, A. Martínez de Ilarduya, M. García-Alvarez, S. Muñoz-Guerra. Third International Symposium Frontiers in Polymer Science, 2013, Spain.

e) *Poly(β,L-Malic Acid) Esterification for Nanoparticle Formation and Drug Encapsulation.* A. Lanz Landázuri, A. Martínez de Ilarduya, J. Portilla-Arias, M. García-Alvarez, S. Muñoz-Guerra. 1st International Conference in Polymers with Special Focus in Early Stage Researchers (Polymar), 2013, Spain.