pH-Responsive diblock copolymers made of ω -pentadecalactone and ionically charged α -amino acids

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Abstract

Two sets of ionically charged polypentadecalactone-polypeptide diblock copolymers (PPDL_x-*b*-PAA_y, with x= 15 or 20 and y ranging from 30 to 200), one containing α -L-glutamic acid (LGA) and the other containing α -L-lysine (LL), were obtained from their respective precursors with the side groups of LGA and LL protected as γ O-benzyl and ϵ N-carbobenzoxymethyl, respectively. The copolymers were semicrystalline with the polyester block crystallized in the usual pseudo-rhombic lattice and the polypeptide in the α -helix or β -sheet conformation depending on the amino acid and the length of the block. Copolymers with PAA blocks with y $\geq$ 80 were water-soluble and they adopted the α -helix conformation in the aqueous medium when they are in the non-ionized state. Both LGA and LL containing copolymers self-assembled in nanoparticles with a size between 150 and 180 nm in diameter. PPDL-*b*-PLGA nanoparticles were able to load DOX with an efficiency of ~70% whereas PPDL-*b*-PLL displayed a noticeable capacity for condensing DNA. In both cases hosting was based on the ionic complexation taking place between the ionized copolymer and the guest compound. Accordingly DOX release rate was found to be noticeably depending on pH.

1. Introduction

Among the polymer materials that are addressed to the design of drug delivery systems, amphiphilic block copolymers are particularly appreciated [1,2]. This is so because the combination of two polymer blocks showing opposite water affinity renders biphasic systems prone to self-assemble in nano-morphologies well suited for encapsulation and transportation of drugs [3]. A distinguished class of these copolymers is that consisting of hydrophobic polyester and hydrophilic polypeptide blocks [4]. Aliphatic polyesters are the polymers of choice because their chemical versatility together with their friendly behavior in physiological environments provide a broad portfolio of materials with appealing properties in the biomedical field [5,6]. On the other hand, polypeptides are widely recognized for their good biocompatibility and biodegradability *in vivo* mediated by specific enzymes whereas they show high stability against chemical hydrolysis [7]. Additionally they are exceptional for their ability of taking up precise secondary conformations [8,9]. Nevertheless it is the pendant functionality provided by certain amino acids that makes polypeptides particularly interesting as building blocks for the synthesis of stimuli-responsive copolymers [10-12].

Many polyester-polypeptide block copolymers are readily attainable by ring opening polymerization of a wide collection of both lactones [13] and α -amino acid *N*-carboxyanhydrides [14]. Representative examples are those made of common aliphatic polyesters coming from medium-size lactones as poly(glycolic acid), polylactides, poly(ϵ -caprolactone) and polycarbonates [15-18]. More recently, macrolactones (rings with 11 or more atoms) have been included as polyester comonomers to generate amphiphilic polyester-polypeptide block and graft copolymers with the hydrophobic moiety displaying properties close to paraffins [19-21]. An outstanding subclass of hybrid polyester-polypeptide copolymers are those made of acid or basic amino acids, namely, aspartic and glutamic acids or lysine, arginine and histidine, which may be negatively or positively charged, respectively, depending on pH. When these

chargeable polypeptides are covalently coupled with neutral polypeptides or synthetic hydrophobic polymers, the physico-chemical behavior of the resulting copolymers, including their conformation and assembling properties, turns out to be highly sensitive to external stimuli, in particular to pH changes. A number of chargeable block copolymers entirely made of α -amino acids [11] as well as those combining polypeptide with a variety of synthetic polymer segments have been reported [22-26] including several examples containing polyester blocks [27-31]. The situation is more precarious when the polyester block is made from macrolactones, *i.e.* lactones consisting in 12 or more atoms (MLs) with no case reported to date. These large size lactones have recently emerged as a new class of building blocks for polymeric functional materials [32]. The polyesters made of MLs have hydrophobic character comparable to paraffins and display a strong tendency to crystallize. These features allow designing amphiphilic copolymer systems with a good hydrophilic-lipophilic balance (HLB) and great shape stability using relatively small amounts of polyester.

We have recently reported on polyester-polypeptide diblock copolymers (PPDL-*b*-pPAA) made of ω -pentadecalactone (PDL) and protected α -amino acids (pAA), either γ -benzyl-L-glutamate (BLG) or ϵ -N-carbobenzoxy-L-lysine (ZLL) [20]. To our knowledge, this is the only example of polymacrolactone-*b*-polypeptide diblock copolymers described in the literature. PPDL-*b*-pPAAs were synthesized by ROP of their respective α -amino acid *N*-carboxyanhydrides initiated by an amino-ended polyester previously produced by enzymatically ROP of PDL. These neutral copolymers were extensively characterized and their capacity for generating nanoparticles was examined. The suitability of PPDL-*b*-pPAA to directly render ionizable copolymers by simple deprotection of their polypeptide moieties was there pointed out as an additional merit of such copolymers. Following our previous work, we wish to report in this occasion on the deprotected PPDL-*b*-PAA diblock copolymers and their potential as biomaterials for drug delivery. The paper covers their synthesis from PPDL-*b*-pPAA, their structure and

thermal properties in the solid state, and their behavior in aqueous medium regarding their self-assembling properties. The capacity of the negatively and positively charged PPDL-*b*-PAA to load hydrophobic drug and to condense DNA respectively by ionic complexation has been preliminary assessed.

2. Experimental section

2.1 Materials

Triphosgene, α -pinene, trifluoroacetic acid (TFA) and HBr/acetic acid, were purchased from Sigma-Aldrich and γ -benzyl L-glutamate (BLG) and ϵ -N-carbobenzoxy-lysine (ZLL) were purchased from Bachem. Anhydrous dimethyl formamide (DMF), tetrahydrofuran (THF), ethyl acetate and heptane were used directly from the bottle under an inert atmosphere. Toluene and chloroform were distilled and dried on 3 Å molecular sieves. ω -Pentadecalactone (PDL) was purchased from Sigma-Aldrich and distilled under vacuum previously to use. 2-(Boc-amino)ethanethiol (BAE) was purchased from Sigma-Aldrich and used without further purification. Novozyme 435 (CALB, *Candida antarctica* Lipase B immobilized on cross-linked polyacrylate beads) was a gift of Novozymes. The received CALB was subjected to drying over molecular sieves under vacuum for 24 h before use. DOX·HCl (98%) was purchased from AK Scientific, Inc. (USA).

2.2 Polymer characterization

^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-300 spectrometer at 25 °C operating at 300.1 and 75.5 MHz, respectively. Compounds were dissolved in deuterated chloroform (CDCl_3) or a mixture of trifluoroacetic acid (TFA) and CDCl_3 , and spectra were internally referenced to tetramethylsilane (TMS). About 10 and 50 mg of sample in 1 mL of solvent were used for ^1H and ^{13}C NMR, respectively. Sixty-four scans were recorded for ^1H , and between 1000 and 10,000 scans for ^{13}C NMR.

Thermogravimetric analysis (TGA) was performed on a Mettler-Toledo TGA/DSC 1 Star System under a nitrogen flow of 20 mL min $^{-1}$ at a heating rate of 10 °C min $^{-1}$ and

within a temperature range of 30-600 °C. The reversible thermal behavior was examined by differential scanning calorimetry (DSC) using a Perkin-Elmer DSC 8000 apparatus. Thermograms were registered from 4-6 mg samples at heating and cooling rates of 10 °C·min⁻¹ under a nitrogen flow of 20 mL·min⁻¹. Indium and zinc were used as standards for temperature and enthalpy calibration.

FTIR measurements were made on a Perkin-Elmer Frontier spectrometer. Spectra in the 4000-450 cm⁻¹ region were obtained from 8 scans with a resolution of 4 cm⁻¹. Molecular weight analysis was performed by GPC on a Waters equipment provided with RI and UV detectors using hexafluoro-2-propanol (HFIP) as eluent. 100 µL of 0.1% (w/v) sample solution were injected and chromatographed with a flow of 0.4 mL·min⁻¹. HR5E and HR2 Waters linear Styragel columns (7.8 mm x 300 mm, pore size 10³-10⁴ Å) packed with crosslinked polystyrene and protected with a pre-column were used. Molar mass averages and their distributions were calculated against PMMA standards. Real time X-ray diffraction (XRD) studies were carried out using synchrotron radiation at the BL11 beamline for non-crystalline diffraction (NCD), at ALBA (Cerdanyola del Vallès, Barcelona, Spain). WAXS and SAXS spectra were recorded simultaneously from powder samples subjected to heating-cooling cycles at a rate of 10 °C min⁻¹. The energy employed corresponded to a 0.10 nm wavelength, and spectra were calibrated with silver behenate (AgBh) and Cr₂O₃.

The conformational analysis of the copolymers in aqueous solution was accomplished by circular dichroism (CD) spectroscopy. Measurements were performed using a Jasco spectropolarimeter J-815) (Centres Científics i Tecnològics, Universitat de Barcelona). Briefly, PPDL-*b*-PAA copolymer solutions were prepared at a concentration of 50 µg·mL⁻¹ in deionized water previously adjusted at the desired pH (2.0 or 10.0). Triplicate CD spectra were recorded using a 10 mm quartz cell at a scanning speed of 10 nm·s⁻¹ in the 190-250 nm range either at constant temperature of 25 °C or at variable temperature in the 10-80 °C range. Dynamic light scattering studies (DLS) were performed using a Zetasizer Nano ZS series Malvern instrument equipped

with a 4 mW He-Ne laser operated at a wavelength of 633 nm. Samples in deionized water were placed in disposable cuvettes thermostated at 25 °C. The non-invasive back-scatter optical arrangement was used to collect the light scattered by the particles at an angle of 173°.

Scanning electron microscopy images were taken with a field-emission JOEL JSM-7001F instrument (JEOL, Japan) from platinum/palladium coated samples.

2.3. Synthesis of precursors and copolymer deprotection

2.3.1 Poly[(ω -pentadecalactone)-*b*-(protected amino acid)s] diblock copolymers (PPDL_x-*b*-pPAA_y)

Poly[(ω -pentadecalactone)-*b*-(γ -benzyl-L-glutamate)] (PPDL_x-*b*-PBLG_y) and poly[(ω -pentadecalactone)-*b*-(ϵ -N-carbobenzoxy-L-lysine)] (PPDL_x-*b*-PZLL_y) diblock copolymers were synthesized as it was previously described in full detail [20]. The set of (PPDL_x-*b*-pPAA_y) copolymers used for generating the charged copolymers studied in this work are listed in Table 1 where their more relevant features in relation to the present study have been included.

Table 1. PPDL_x-*b*-pPAA_y copolymers used as precursors in this work.^a

Copolymer ^b	Copolymer ^c [PDL]/[AA]	M_n^d (g·mol ⁻¹)	T_g^e (°C)	T_m^e (°C)	T_{LC}^e (°C)
PPDL ₁₅ - <i>b</i> -PBLG ₃₀	15/33	10,500	21	90	109
PPDL ₁₅ - <i>b</i> -PBLG ₆₀	15/56	15,500	21	88	109
PPDL ₁₅ - <i>b</i> -PBLG ₈₀	15/84	21,600	21	88	119
PPDL ₁₅ - <i>b</i> -PBLG ₁₈₀	15/187	44,200	20	88	120
PPDL ₂₀ - <i>b</i> -PZLL ₃₀	20/32	12,800	21	83	118
PPDL ₂₀ - <i>b</i> -PZLL ₇₀	20/68	22,300	21	84	-
PPDL ₂₀ - <i>b</i> -PZLL ₁₀₀	20/98	30,000	20	84	-
PPDL ₂₀ - <i>b</i> -PZLL ₁₉₀	20/190	54,300	20	90	~140

^aData taken from reference 20.

^bSubscripts indicate the number-average degrees of polymerization of the two blocks with y values being rounded to next ten.

^cCopolymers composition expressed as the number of units of each block as determined by ¹H NMR.

^dNumber-average molecular weight of copolymers determined by ¹H NMR. Molar mass dispersities ($\bar{D}$) of these copolymers determined by GPC in HFIP are in the 1.2-2.2 range.

^eGlass transition (T_g), PPD L block melting (T_m), and mesophasic transition (T_{LC}) temperatures measured by DSC.

2.3.2. Poly[(ω -pentadecalactone)-*b*-(α -amino acid)] diblock copolymers (PPDL_x-*b*-PAA_y).

Poly[(ω -pentadecalactone)-*b*-(L-glutamic acid)] diblock copolymers (PPDL_x-*b*-PLGA_y). The benzyl carboxylate group of the polypeptide block of PPDL_x-*b*-PBLG_y copolymers was split by treatment with hydrobromic acid. 220 mg of the esterified copolymer were dissolved in TFA (2 mL) and then 1 mL of 33% (w/w) HBr in acetic acid was added slowly to this solution at 0 °C. After 3 h of reaction, the mixture was poured into an excess of diethyl ether and the precipitate was recovered by centrifugation. The carboxylic-free PPDL_x-*b*-PLGA_y was washed twice with fresh diethyl ether and then dissolved in a 0.5 M NaHCO₃ aqueous solution. Other sodium salts generated in the reaction were removed by dialysis against distilled water for 72 h at room temperature using MW-CO 2.0 kDa membranes. The deprotected copolymer PPDL_x-*b*-PLGA_y in the sodium salt form was recovered as a white powder after removing the water by rotaevaporation. Yield: 60-74%.

Poly[(ω -pentadecalactone)-*b*-(L-lysine)] (PPDL_x-*b*-PLL_y). Deprotection of PPDL_x-*b*-PZLL_y copolymers by applying the same procedure described above for PPDL_x-*b*-PBLG_y led to the amino-free copolymers PPDL_x-*b*-PLL_y. Yield: 65-72%

2.4. Nanoparticle formation and drug loading

5 mg of PPDL₁₅-*b*-PLGA₈₀ copolymer was solubilized in 4.0 mL of deionized water and left under stirring for 10 min and the measured pH was 7.0. Then, DOX·HCl (4, 3, 2, or 1 mg) dissolved in deionized water (1.0 mL) was added dropwise into the polymer-solution and the mixture was stirred for 12 h and then dialyzed (MWCO 2000) for 24 h against distilled water to remove the free DOX. The dialyzed mixture was lyophilized and the dried solid used for Drug Loading Efficiency (DLE) and Drug Loading Content (DLC) of DOX-NPs calculations by applying the following equations:

$$DLE\% = \frac{\text{Mass of the drug in NPs}}{\text{Mass of the drug in feed}} \times 100 \quad \quad DLC\% = \frac{\text{Mass of drug in NPs}}{\text{Mass of NPs}} \times 100$$

3. Results and discussion

3.1. Synthesis of ionic PPDL_x-*b*-PAA_y diblock copolymers.

The PPDL_x-*b*-pPAA_y diblock copolymers constituted by PDL and protected amino acids, either γ -benzyl-L-glutamate or ϵ -N-carbobenzoxy-L-lysine, were recently reported by us, and the chemical route followed for their synthesis is depicted in Scheme S1 of the SI file. These copolymers have been used in the present work for preparing their corresponding deprotected PPDL_x-*b*-PAA_y copolymers. Thus the removal of the benzyl ester group in PPDL_x-*b*-PBLG_y and the benzyloxycarbonyl group in PPDL_x-*b*-PZLL_y by treatment with HBr led to the PPDL_x-*b*-PLGA_y and PPDL_x-*b*-PLL_y copolymers, respectively, in which the polypeptide block bears either one free carboxylic side group or one free amino group in every repeating unit. The reactions involved in such modifications are formulated in Scheme 1 and the results attained are given in Table 2. Yields were in the 60-75% range with losses being mainly attributable to limitations in the recovery and purification of the final product. Number-average molecular-weights determined by NMR end-group analysis of deprotected copolymers were close to the values calculated by subtracting the protecting group mass from the M_n of their respective protected copolymers.

Scheme 1. Treatment applied for amino acid deprotection of diblock copolymers.

Differences between M_n values obtained by the two methods were less than 5% for the case of PPDL_x-*b*-PLL_y and around 12-13% for PPDL_x-*b*-PLGA_y indicating that splitting of the main chain caused by the acidic treatment must be negligible in the

former and of little significance in the latter. Additionally, the ^1H NMR analysis proved that full conversion was attained in the hydrolysis of the protected groups and also ascertained the composition of the deprotected copolymers. Representative spectra of the two series are shown in Figure 1 with indication of the assignments for all the observed signals. Additional examples are given in Figure S1 of the SI file.

Table 2. Yield, M_n and thermal properties of deprotected $\text{PPDL}_x\text{-}b\text{-PAA}_y$ copolymers.

Copolymer ^a	Yield (%)	M_n^b ($\text{g}\cdot\text{mol}^{-1}$)	TGA ^c			DSC ^d					
						1 st heating		1 st cooling		2 nd heating	
			$^{\circ}T_d^a$ $^{\circ}\text{C}$	$^{\text{max}}T_d$ $^{\circ}\text{C}$	R_w %	T_m $^{\circ}\text{C}$	ΔH $\text{J}\cdot\text{g}^{-1}$	T_c $^{\circ}\text{C}$	ΔH $\text{J}\cdot\text{g}^{-1}$	T_m $^{\circ}\text{C}$	ΔH $\text{J}\cdot\text{g}^{-1}$
PPDL ₁₅	90	3600	285	430 470	1	90	151	75	-119	90	123
PPDL ₁₅ - <i>b</i> -PLGA ₃₀	70	7500 (8450)	270	370	21	89	72	70	-23	90	29
PPDL ₁₅ - <i>b</i> -PLGA ₆₀	70	10,500 (11,850)	290	325	40	86	89	68	-13	90	15
PPDL ₁₅ - <i>b</i> -PLGA ₈₀	74	14,000 (15,950)	295	315	40	85	84	65	-4	92	3
PPDL ₁₅ - <i>b</i> -PLGA ₁₈₀	60	27,400 (31,100)	300	300	70	100	110	n.o.	n.o.	n.o.	n.o.
PLGA ₅₀	70	6550	290	300	49	74	51	-	-	-	-
PPDL ₂₀	94	4800	285	430 470	1	90	151	75	-119	90	123
PPDL ₂₀ - <i>b</i> -PLL ₃₀	72	8500 (8900)	228	470	12	91	112	72	-81	85	70
PPDL ₂₀ - <i>b</i> -PLL ₇₀	70	13,200 (13,500)	272	450	10	87	65	78	-11	89	13
PPDL ₂₀ - <i>b</i> -PLL ₁₀₀	70	17,400 (17,700)	180	300	15	85-90	35	78	-6	91	10.0
PPDL ₂₀ - <i>b</i> -PLL ₁₉₀	65	28,800 (29,150)	300	340	18	85-90	7	72	-1	91	0.9
PLL ₅₀	75	6500	270	325	16	85	48	-	-	-	-

^aSubscripts have the same meaning as in Table 1.

^bNumber-average molecular weight determined by ^1H NMR. In parenthesis the molecular weight (rounded to ten) calculated for the composition given in Table 1.

^cOnset temperature for 5% weight loss ($^{\circ}T_d$) and maximum rate ($^{\text{max}}T_d$) decomposition temperatures measured in the TGA analysis performed under inert atmosphere. R_w : weight (%) remaining after heating at 600 $^{\circ}\text{C}$.

^dMelting (T_m and ΔH_m) and crystallization (T_c and ΔH_c) temperatures and enthalpies measured by DSC at heating (first and second run) and cooling.

Figure 1. ^1H NMR spectra of $\text{PPDL}_{15}\text{-}b\text{-PLGA}_{80}$ in CDCl_3/TFA (a) and $\text{PPDL}_{20}\text{-}b\text{-PLL}_{100}$ in CDCl_3/TFA (b) and water (b').

3.2. Thermal properties of the PPDL_x-*b*-PAA_y diblock copolymers

The TGA traces of the deprotected PPDL_x-*b*-PAA_y diblock copolymers registered under a nitrogen atmosphere within the 20-600 °C temperature range as well as their derivative curves are accessible in the SI file as Figure S2. The most significant decomposition parameters of the two series provided by TGA analysis are listed in Table 2. Compared to the protected copolymers [20], the thermal stability in overall increased after deprotection. In fact, onset temperatures are observed well above 200 °C and bulk decompositions take place around 300 °C. The derivative curves showed that decomposition evolved through a rather complicate process consisting in several steps, the last one taking place at temperatures not far from 500 °C which probably corresponds to the decomposition of the PPDL block. What is really different is the amount of weight remaining after heating at 600 °C which appears to be much higher in the case of the deprotected copolymers with values reaching up to 70% in the case of PPDL₁₅-*b*-PLGA₁₈₀. No doubt such large differences must be due, at least in part, to the ionic nature of PPDL_x-*b*-PAA_y copolymers.

The DSC heating and cooling traces recorded from the PPDL_x-*b*-PLGA_y and PPDL_x-*b*-PLL_y copolymers are shown in Figure 2, and the thermal data provided by this analysis are gathered in Table 2.

Figure 2. DSC traces recorded at heating from pristine samples of PPDL_x-*b*-PLGA_y (a) and PPDL_x-*b*-PLL_y (b) diblock copolymers.

The heating DSC traces displayed by these copolymers have in common the presence of the melting peak of the PPDL block in the 80-90 °C temperature range followed by a more or less prominent endotherm that, according to related antecedents, it could be associated to a conformational transition taking place in the polypeptide block. The occurrence of conformational transitions in the unprotected PLGA and PLL blocks of polypeptide-based block copolymers has been reported previously by other authors [33] and is consistent with the presence of ordered conformations. One or two exothermal peaks corresponding to one or two-step crystallization of the PPDL block were observed in the cooling traces. A critical inspection of Table 2 reveals that T_m values recorded at the first heating fluctuates inconsistently along the two series, which is in contrast with the rather steady variation and peak sharpness that is observed for such transition when recorded at the second heating (see Figure S3 in the SI file). These results indicate that crystallization of the PPDL must be sensitively affected by the history of the samples, a fact that is uncontrolled when they come directly from synthesis but that is essentially the same when they are crystallized from the melt.

3.3. Solid-state structure of the PPDL_x-*b*-PAA_y copolymers

FTIR analysis of powder samples of PPDL_x-*b*-PAA_y was performed to get insight the molecular arrangement that is adopted by the polypeptide block in these copolymers in the solid-state. The spectra of the two series are compared in Figure 3 which shows intensity variations in the characteristic absorptions of the copolymers that are consistent with composition. Thus the broad band appearing around 3500 cm⁻¹ due to the stretching vibration of O-H or/and N-H bonds increased steadily with the AA/PDL ratio whereas exactly the opposite happened with the 2950-2800 cm⁻¹ and 1750 cm⁻¹ bands arising from the methylene and carbonyl ester groups present in the PPDL block. Additionally a close inspection of the absorption pattern recorded in the 1700-1500 cm⁻¹ region revealed significant differences not only between the two series but

also for the different compositions within each of them. The spectra of PPDL₁₅-*b*-PLGA_y copolymers show a band at 1645-1650 cm⁻¹ with transmittance practically constant along the series. This band is commonly assigned to the Amide I band of the PLGA block in α -helix conformation although the presence of random coil arrangement cannot be discarded. On the other hand, the PPDL₂₀-*b*-PLL_y series displays the Amide I band at ~1625 cm⁻¹ whose intensity increased with y. This band is characteristic of the β -sheet form and its presence reveals that the PLL block in these copolymers must be partially arranged with chains in almost extended conformation and hydrogen bonded in a proportion that increases with the length of the polypeptide block.

Figure 3. FTIR of PPDL_x-*b*-PAA_y diblock copolymers. In a' and b' plots, the 1800-1500 cm⁻¹ region has been enlarged for a better comparison of characteristic absorptions of the α -helix and β -sheet forms.

The structure of the PPDL-*b*-PAA copolymers in the solid-state was then examined by XRD at variable temperature using synchrotron radiation and covering both WAXS and SAXS regions. The WAXS scattering profiles recorded for PPDL₁₅-*b*-PLGA₈₀ and PPDL₂₀-*b*-PLL₁₀₀ at both heating and cooling in the 10-150 °C temperature range are plotted in Figure 4. The reflections at 0.41 and 0.37 nm indexed as 110 and 200, which are characteristic of the pseudo-rhombic monoclinic unit cell of PPDL with approximate dimensions $a = 0.75$ nm, $b = 0.5$ nm, and $c = 2.0$ nm and $\alpha = 90.0^\circ$ [34,35], are apparent in the profiles of the two copolymers recorded at 10 °C. Upon heating the two reflections were initially unaltered but they disappeared abruptly when temperature reached the proximities of 90 °C. The same behavior was displayed by the weak reflection at ~0.71 nm observed in the diffraction profiles of PPDL₂₀-*b*-PLL₁₀₀ which could be tentatively indexed as the 101 of the PPDL crystal lattice. The two $hk0$ reflections were fully recovered upon cooling when temperature arrived to around 65 °C and 80 °C for the LGA and the LL containing copolymers, respectively. These results are in full agreement with those obtained by DSC confirming that the PPDL block was crystallized in all the PPDL_x-*b*-PAA_y copolymers, and that this block is able to recrystallize from the melt at an undercooling that is significantly larger for the LGA containing copolymers. Since no sign of any other reflection is observed in the WAXS profiles recorded from PPDL₁₅-*b*-PLGA₈₀, it should be concluded that the order attained by the PLGA blocks in this copolymer must be low. Conversely, the WAXS profiles produced by PPDL₂₀-*b*-PLL₁₀₀ show additional reflections at 1.4 and 0.81 nm which could be attributed to the β -sheet structure in which the PLL block seems to be arranged as it was clearly revealed by FTIR. It is worth noting that the scattering due to the β -sheet form phase was attenuated at temperatures above melting of the PPDL phase so that the 0.81 reflection was the only remaining at 150 °C. After cooling, the β -form was not recovered whereas the PPDL crystallized so that its initial diffraction pattern became well reproduced. At difference with what was reported for the protected

PPDL-*b*-pPAA copolymers, no discrete scattering was observed in the SAXS profiles produced by the deprotected PPDL-*b*-PAA copolymers indicating that they are not assembled in any ordered nanostructure, at least below the 60 nm scale.

Figure 4. Evolution of the WAXS profiles of PPDL₁₅-*b*-PLGA₈₀ (a,a') and PPDL₂₀-*b*-PLL₁₀₀ (b,b') recorded at heating and cooling over the 10-150 °C at a rate of 10 °C·min⁻¹. The reflection at 1.4 nm that is cited in the text is hardly seen in b). For a better visualization, an enlarged region has been reproduced in Figure S4 of the SI. The peak observed at 0.34 nm is an artefact of unknown origin that was produced during the register of data.

3.4. Properties in water solution and self-assembling of PPDL_x-*b*-PAA_y.

The protected PPDL_x-*b*-pPAA_y diblock copolymers were non-water soluble as it could be reasonably expected from the presence of the hydrophobic groups used for protection. Conversely, the solubility in water of the deprotected PPDL_x-*b*-PAA_y copolymers is strongly dependent on both the x/y ratio and the ionic state of the polypeptide counterpart. In the non-ionized form none of these copolymers could be solubilized in water but PPDL₁₅-*b*-PLGA₈₀ and PPDL₁₅-*b*-PLGA₁₈₀ as well as PPDL₂₀-*b*-PLL₁₀₀ and PPDL₂₀-*b*-PLL₁₉₀ became water-soluble when they were in the sodium and

hydrobromide salts, respectively. The high contrast between the strong hydrophobic nature of the polyester block and the ionically charged polypeptide block in these copolymers make them to display a markedly amphiphilic behavior that should be reflected in their capacity to self-assemble in aqueous medium. The conformational preferences of the water-soluble PPDL_x-*b*-PAA_y copolymers when dissolved in water, their critical micelle concentrations (*cmc*), and the main features of the nanoparticles that they are able to form, are compared in Table 3.

Table 3. Features of the water-soluble PPDL_x-*b*-PAA_y diblock copolymers in aqueous medium

Copolymer	CD		DLS			
	Secondary structure		<i>cmc</i> (mg·mL ⁻¹)	<i>D</i> (nm)	<i>PDI</i>	ζ (mV)
	pH=2	pH=10				
PPDL ₁₅ - <i>b</i> -PLGA ₈₀	helix	coil	0.37	146	0.26	-4.12
PPDL ₁₅ - <i>b</i> -PLGA ₁₈₀	helix	coil	0.79	153	0.26	-45.3
PPDL ₂₀ - <i>b</i> -PLL ₁₀₀	coil	helix	0.26	180	0.12	39.5
PPDL ₂₀ - <i>b</i> -PLL ₁₉₀	coil	helix	0.30	176	0.24	70.8

Firstly the determination of the *cmc* was carried out by measuring the variation in light scattering intensity with polymer concentration in deionized aqueous solution. As expected, the resulting plots (see Figure S5 in SI file) clearly indicated a sharp change in the slope of the straight line at a concentration that increased with the PAA to PPDL ratio. Secondly the occurrence of regular conformations of PPDL_x-*b*-PAA_y in aqueous solution and their dependence on pH and temperature were investigated by CD spectroscopy at concentrations well below the *cmc*. The CD traces recorded for PPDL₁₅-*b*-PLGA₈₀ and PPDL₂₀-*b*-PLL₁₀₀ under different conditions are shown in Figure 5. The maxima dichroic pair appearing at 212 and 222 nm with negative sign, which is the profile characteristic of α -helix, became well appreciated in these compounds at pH 2 and 10 respectively in agreement with to their non-ionized states. As it should be expected, the helical dichroism profile disappeared when pH changed from acid to basic in the former and in the opposite sense in the latter. The heating effect was

similar in both cases with the helical structure being progressively disrupted as temperature increased.

Figure 5. CD spectra of $\text{PPDL}_x\text{-b-PAA}_y$ diblock copolymers in aqueous solution at a concentration of $50 \mu\text{g}\cdot\text{mL}^{-1}$.

The DLS plots of the aqueous solutions of the $\text{PPDL}_x\text{-b-PAA}_y$ copolymers at concentrations above the *cmc* are shown in Figure 6 (a and b). These plots evidence the presence of aggregates of nanometric size with a diameter that is dependent on the amount of dissolved copolymer. The increase in size of micelle-like objects with increasing copolymer concentration, as it is here observed, has been previously reported for both block and graft poly(caprolactone-L-lysine) copolymers [30,37]. It is argued that larger plurimolecular aggregates must be formed as the copolymer adopts a more extended conformation in solution. Bimodal distributions were occasionally obtained indicating that aggregation take place in more than one specific manner. The

ζ -potential displayed by these nanoparticles was positive or negative for LL and LGA containing copolymers respectively in agreement with the sign charge of the polypeptide present in each case. In both cases, the absolute value of ζ increased with the length of the polypeptide block, as expected. In order to get insight in the arrangement adopted by the polypeptide blocks in the PDDL_x-*b*-PLL_y micelles compared to that observed in bulk, a sample of PDDL₂₀-*b*-PLL₁₀₀ was prepared by freeze-drying one 1 mg·mL⁻¹ aqueous solution and then examined by FTIR. The Amide I region of the spectrum produced by this sample displayed the bands characteristic of the β -sheet form with chains arranged mainly in antiparallel (1690 and 1620 cm⁻¹) but with a significant contribution of the form made of chain arranged in a parallel array (1640 cm⁻¹).

Figure 6. DLS plots of aqueous solutions of PPDL₁₅-*b*-PLGA₁₈₀ (a) and PPDL₂₀-*b*-PLL₁₉₀ (b) at different concentrations, and SEM images of the nanoparticles (a' and b') left upon water evaporation of the lowest concentration solutions.

The morphology of the nanoaggregates formed in aqueous solution at concentrations above *cmc* was examined by SEM. The significance of the resulting nanoparticles in terms of size is not reliable since aggregation is highly sensitive to concentration and therefore it will be enhanced during the evaporation of the drop. Nevertheless the SEM images (Figure 6a' and 6b') showed homogeneous populations of nanoparticles well separated from each other that could be clearly differentiated.

3.5. Preliminar evaluation of PPDL_x-*b*-PAA_y copolymers as drug nanocarriers

3.5.1. PPDL_x-*b*-PLGA_y copolymers: Doxorubicin loading and release

Doxorubicin (DOX), a widely-known antineoplastic compound [37], was used as model to evaluate the potential of the anionic PPDL_x-*b*-PLGA_y copolymers to be used as drug delivery systems. Two LGA-containing copolymers differing in the length of the PLGA block were selected for this study, namely PPDL₁₅-*b*-PLGA₈₀ and PPDL₁₅-*b*-PLGA₁₈₀. In these copolymers, ionic coupling between DOX and the carboxylate side groups of the LGA units should be expected to be the main mechanism responsible for entrapping.

The DLS experimental traces registered from (PPDL_x-*b*-PLGA_y)-DOX conjugates for LGA/DOX molar ratios of 5:1 and 8:1 and for the blanks (pristine copolymers) nanoparticles are shown in Figure 7 and their diameters (*D*), polydispersity indexes (*PDI*) and zeta potentials (ζ) measured by this technique are compared in Table 4. In both cases, the monomodal traces observed for the unloaded NPs made of pristine copolymers became split when they were loaded with DOX. The two new signals corresponded to NPs sizes smaller and greater than that of the unloaded ones, and the negative zeta potential significantly decreased upon DOX loading in both cases.

Figure 7. DLS profiles of DOX-charged NPs made of PPDL₁₅-*b*-PLGA_y diblock copolymers.

Table 4. Characterization of DOX-loaded nanoparticles.

Copolymer or conjugate	LGA/DOX (mol/mol)	<i>D</i> (nm)	<i>PDI</i>	ζ (mV)
PPDL ₁₅ - <i>b</i> -PLGA ₈₀	-	360	0.32	-39
(PPDL ₁₅ - <i>b</i> -PLGA ₈₀)-DOX	5/1	160/490	0.53	-20
PPDL ₁₅ - <i>b</i> -PLGA ₁₈₀	-	460	0.14	-45
(PPDL ₁₅ - <i>b</i> -PLGA ₁₈₀)-DOX	8/1	140/550	0.60	-29

To assess the influence of the feed composition on coupling efficiency, a set of (PPDL₁₅-*b*-PLGA₈₀)-DOX conjugates were prepared using different LGA/DOX ratios. Copolymer/drug mixtures prepared at the selected ratios were dialyzed against water to remove non-associated DOX and the UV absorption of the dialysate at 480 nm was measured to estimate the amount of DOX that remained attached to the copolymer. Additionally, the residue recovered upon lyophilization of the dialysate was examined by ¹H NMR using external calibration to determine the absolute amount of DOX present in the nanoparticles (an illustrative spectrum is shown in Figure S7 in the SI file). The values obtained for DLE (Drug Loaded Efficiency) and DLC (Drug Loaded Content), as well as for the zeta-potential are graphically shown in Figure 8 for the different tested LGA/DOX ratios. For low values the two parameters increased with the proportion of drug used to form the conjugate but this trend vanished for ratios lower than 5/1. All DOX-loaded particles exhibited a negative surface charge around 20 mV lower than

that of the unloaded which is consistent with the neutralization of part of the LGA carboxylate groups by the loaded drug molecules [38].

Figure 8. PPDL₁₅-b-PLGA₈₀-DOX conjugates obtained at different LGA/DOX ratios. a) DLE and DLC, and b) zeta potential.

Release of DOX from the (PPDL₁₅-b-PLGA_y)-DOX conjugates incubated in aqueous medium is expected to happen as a consequence of the dissociation of the ionic complex, a process that must be pH-dependent. DOX-loaded NPs prepared from PPDL₁₅-b-PLGA₈₀ using a feed with a LGA/DOX ratio of 5/1 were chosen to evaluate the release of DOX at 37 °C at three different pHs, *i.e.* in PBS, pH 7.4, citrate-phosphate buffer, pH 4.2 and pH 2.0 attained by adding HCl. The plots of the cumulative DOX release obtained are shown in Figure 9. At neutral pH the release of the drug happened fast in the first several hours of incubation to slow down later to the point that the 100% of delivery was not reached even after 50 h. On the contrary, the release at pH 4.2 took place at a noticeably high rate than at pH 7.4 as it could be reasonably expected from the enhancing effect of pH on the dissociation of the LGA-DOX ionic complex. At pH 2 DOX was almost fully released in less than 5 h. This effect was clearly evidenced in the ¹H NMR spectra shown in Figure 10 which were registered from PPDL₁₅-b-PLGA₈₀ copolymer and its DOX conjugate at neutral and

acidic aqueous media. It is noteworthy that signals from DOX were almost undetectable at neutral pH whereas they became clearly visible at pH 2 indicating that an extensive dissociation of the complex must occur in the acidic medium. Similar experiments carried out with PPDL₁₅-*b*-PLGA₁₈₀ afforded similar results (Figure S7 in the SI file) indicating that the influence of the PLGA block length on DOX release is not significant.

Figure 9. Cumulative release (%) of DOX from loaded micelles of PPDL₁₅-*b*-PLGA₈₀ (a) and PPDL₁₅-*b*-PLGA₁₈₀ (b) diblock copolymers at pH 7.4, 4.2 and 2. The release study was performed using the dialysis method and DOX concentration was estimated by UV absorption at 480 nm. Data are given as mean $\pm$ SD of triplicates.

Figure 10. ^1H NMR spectra (D_2O) of the $\text{PPDL}_{15}\text{-}b\text{-PLGA}_{80}$ copolymer and $(\text{PPDL}_{15}\text{-}b\text{-PLGA}_{80})\cdot\text{DOX}$ conjugate at neutral and acidic pH.

3.5.2. PPDL_x-*b*-PLL_y copolymers: DNA complexation

The formation of complexes between LL-containing copolymers and DNA was examined using the PPDL₂₀-*b*-PLL₁₀₀ copolymer and salmon testes DNA (stDNA, $M_w \sim 2,000$ bp) in aqueous medium. The polyplexes were prepared at room temperature using deionized water by addition of the copolymer solution to that of DNA and subsequent incubation of the mixture for 20 min. A range of N to P ratios (N/P, mol/mol) going from 30 to 3 was tested. N/P values were determined by considering that the PPDL₂₀-*b*-PLL₁₀₀ and DNA mass per N and P atom is $225 \text{ g} \cdot \text{mol}^{-1}$ and $325 \text{ g} \cdot \text{mol}^{-1}$, respectively. The polyplex solution was subjected to DLS analysis and scattering data compared to those obtained for the pristine copolymer. As it is seen in Figure 11, the original size of copolymer nanoparticles of ~ 250 nm decreased with addition of moderate amounts of DNA (N/P = 30) down to ~ 150 nm indicating that complexation with concomitant condensation of the DNA molecule has taken place. Further addition of DNA entailed a steadily increase in particle size to reach a diameter near 400 nm for N/P = 3. This is consistent with previous observations reporting that the largest aggregates are formed for N/P values close to 1 [39]. Polyplex formation also entailed a decrease in the positive zeta-potential of the copolymer for all compositions, as it should be expected from the charge compensation that must take place upon coupling. However the variation of ζ with composition does not display a logical trend. It decreased about 10 mV for small amounts of DNA (N/P = 30 and 16) and recovered almost totally for N/P = 10. From this value onwards ζ decayed slightly but steadily for increasing content in DNA.

Figure 11. Diameter (a) and zeta potential (b) of the (PPDL₂₀-b-PLL₁₀₀)·DNA polyplexes.

4. Conclusions

Diblock copolymers (PPDL_x-b-PAA_y), either negatively or positively charged, made of polypentadecalactone (PDL) and L-polyglutamic acid (PLGA) or L-polylysine (PLL), respectively, have been successfully prepared. Neutral copolymer precursors obtained by sequential ROP of PDL and amino acid NCA, in which LGA or LL units were duly protected, have shown to be appropriate for this synthesis. Deprotection was easily accomplished to render copolymers with the same PDL/AA composition as the parent precursors. In all PPDL_x-b-PAA_y copolymers, the PPDL block was crystallized and the polypeptidic block was arranged in α -helix or β -sheet conformation depending on the amino acid and the length of the block. These copolymers displayed water solubility for long amino acid blocks in the ionized state and showed helix-coil transition in aqueous solution mediated by pH changes. PPDL_x-b-PAA_y copolymers were able to self-assemble in nanoparticles with sizes between 100 and 200 nm with presumable good stability due to the tight effect provided by the polyester core. The peripheral location of the ionic block made these nanoparticles particularly suitable for anchoring charged molecules by ionic coupling mechanism. DOX was entrapped in the PLGA-based copolymer particles with a loading efficiency near to 80% to attain contents of the drug up to 20% and it was released in hours at a pH-dependent rate. A preliminary

examination of the capacity of the PLL-based copolymers to couple DNA revealed the formation of nanometric ionic polyplexes with apparent condensation for high copolymer/biopolymer ratios.

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Supporting information content

Scheme of the synthesis of copolymer precursors, NMR of copolymers, TGA of copolymers, second heating DSC of copolymers, enlarged WAXS region of PPDL₂₀-*b*-PLL₁₀₀, DLS intensity vs concentration plots used for *cmc* determinations, FTIR spectra of PPDL₂₀-*b*-PLL₁₀₀ (powder and freeze-dried micelles), NMR spectra of (PPDL₁₅-*b*-PLGA₈₀)-DOX conjugates.

Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to time limitations.

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