Block and graft copolymers made of 16-membered macrolactones and L-alanine: A comparative study

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ABSTRACT

Block and graft poly(macrolactone)-poly(α -amino acid) copolymers made of L-alanine and pentadecalactone or globalide respectively, were prepared. A sequential ROP copolymerization route consisting of two stages, the first devoted to the preparation of the amino-functionalized poly(macrolactone) and the second to the amino-initiated polymerization of Ala-NCA, was followed for the synthesis of both types of copolymers. Poly(L-alanine) segment lengths were accurately controlled by adjusting the macroitiator/Ala NCA ratio used for reaction in the second stage. Block copolymers were semicrystalline with the poly(pentadecalactone) block well crystallized in a separate phase and the poly(α -amino acid) block arranged in either the α -helical or β -sheet structure in a ratio that was depending on composition and temperature. Graft copolymers were amorphous but with the poly(α -amino acid) side chains arranged in a more or less regular conformation. Nanoparticles with a diameter of around 300 nm and moderate positive Z-potential could be obtained from the block copolymers by self-assembling in water whereas graft copolymers were unable to render recognizable objects of nanometer-dimension under similar conditions.

INTRODUCTION

Block and graft copolymers in which homogeneous monomer sequences are incompatible and therefore able to spontaneously self-assemble in organized arrangements, are the preferred compounds for building nanostructured polymeric systems.^[1-3] In this regard, amphiphilic copolymers made of hydrophilic and hydrophobic segments are particularly interesting given their capacity to form biphasic nanoparticles including compact, hollow and core-shell objects, when they are free to self-assemble in aqueous media.^[4-6] As a subfamily of heterogeneous copolymers, those integrated by aliphatic polyester and poly(α -amino acid) segments are receiving great attention in these last years due to the outstanding assortment of properties that may be attained by such combination. As a matter of fact, this paper deals with diblock and graft copolymers made of polyesters derived from macrolactones (ML) and the poly(α -amino acid) poly(L-alanine) (PALa).

Poly(α -amino acid)s are biocompatible polymers that exist in a wide variety of chemical structures, many of them readily accessible by ring-opening polymerization (ROP) of α -amino acid *N*-carboxyanhydrides.^[7] These polymers display high conformational versatility and may adopt different structures depending on the surrounding conditions.^[8] Aliphatic polyesters are mostly non-functional synthetic polymers usually lacking molecular activity but displaying an enormous potential for drug delivery applications.^[9-11] These polyesters can be synthesized by relatively simple polymerization procedures, the most used one being the ROP of lactones.^[12] Polyesters generally display a good biocompatibility and are more or less vulnerable to enzymatic degradation depending mainly on their hydrocarbon/ester ratio. A good number of polyester-poly(α -amino acid) block copolymers have been described in the literature, and their capacity to form nanoparticles with potential utility as drug carrier and delivery systems has been

extensively explored.^[13-17] The number of cases dealing with polyester-poly(α -amino acid) graft copolymers that have been studied appear to be much lower,^[18-21] in particular when grafting is made on the polyester counterpart. In both cases, diblock and graft systems, the polyester invariably derived from a medium-size lactone (up to 11 atoms) such as ϵ -caprolactone or L-lactide, and the polypeptide was made of a three-functional α -amino acid such as aspartic or glutamic acid, lysine or serine.

The polyesters made of macrolactones (ML, large-size lactones containing more than 11 atoms) have a hydrophobic character comparable to paraffins, and their potential for the design of biodegradable carriers suitable for drug dispensing applications has been extensively explored along the last decade.^[22-25] However ML-based hybrid copolymers have been scarcely studied and the number of poly(macrolactone-co- α -amino acid) copolymer systems known to date reduces to a couple of examples recently reported by us.^[26-27] In these works, pentadecalactone (PDL) and globalide (GI) were the MLs chosen for the synthesis of block and graft copolymers, respectively, and the α -amino acids were L-glutamic and L-lysine in both cases. The copolymers were prepared by enzymatic ROP of the ML and subsequent coupling by amino-initiated ROP of the conveniently protected α -amino acid. By this method, the length of the blocks and grafted chains of the copolymers could be precisely adjusted. Most of these copolymers were shown to be able to self-assemble in well-shaped nanoparticles with a diameter in the ~200-400 nm range and displayed negative zeta-potential.

L-Alanine is a neutral α -amino acid that has a strong helix-stabilizing effect when incorporated in a polypeptide chain. The small size of the methyl side chain of L-alanine allows high chain flexibility while retaining enough bulk to stabilize the secondary structure through side-chain/side-chain interactions and van der Waals forces. In polymer design, L-alanine has been mostly copolymerized with hydrophilic macromonomers such as

poly(ethylene glycol)^[28-32] to build amphiphilic block copolymers showing nanophase separation. Although L-alanine is catalogued as a hydrophobic α -amino acid, the capacity of the amide group to be hydrogen-bonded with itself or directly to water enables this amino acid to play a relatively hydrophilic function when inserted in highly hydrophobic molecules. Thus block copolypeptides made of L-alanine and γ -benzyl L-glutamate have been synthesized and described to be structurally heterogeneous with the two integrating amino acids taking up the β -sheet and the α -helical structure, respectively.^[33,34] Furthermore L-alanine is known to play a critical role in regulating the secondary structure of spider silks polypeptides due to the influence exerted by the poly(L-alanine) domains arranged in β -sheet form.^[35,36] Copolymers based on the combination of polyalanine and polyester sequences are rare so that only L-lactide^[14] and ϵ -caprolactone^[15] have been used so far as comonomers of L-alanine in the synthesis of block copolymers. On the other hand, L-alanine has been recurrently used for grafting water-soluble polymers^[37] and biopolymers^[38,39] with the aim at making them more water-resistant. To our knowledge, neither block nor graft copolymers made of L-alanine and macrolactones have been investigated so far.

In the copoly(ester-peptide) block structure, the chemical structure of the polyester segment remains essentially unaltered upon copolymerization so its thermal and crystalline behavior is expected to be not far from those of the homopolyester. Conversely, in the graft copoly(ester-peptide) the polyester chain is modified by multiple side chain insertions in the backbone that severely distort its chemical regularity. As a consequence the thermal properties of the grafted polyester become largely changed and its crystallinity is usually lost. Such differences between block and graft copolymers are expected to be clearly reflected in their self-assembling properties and in consequence in their capacity to generate nano-objects with a well-delineate biphasic structure. In this work the utility of the

sequential ROP synthesis to produce well-defined heterogeneous copolymers is applied to the preparation of block and graft copolymers made of L-alanine and either PDL or GI, and their structure, thermal properties and capacity to form self-assembled nanoparticles are assessed through a comparative study. PDL and PGI are two macrolactones sharing the same number of carbons and differing only in the presence of one double bond in the latter. The polyesters generated by the two lactones, PPDL and PGI respectively, are highly crystalline and exhibit almost the same hydrophobicity. PPDL may be ended functionalized whereas PGI is suitable for polyfunctionalization as it is required for grafting. L-Alanine is the amino acid of choice because it does not need to be blocked for NCA ROP and it is able to adopt either α -helical or β -sheet structure. Since crystallinity and conformation of the two counterparts play a crucial role in the eventual arrangement adopted by the copolymer, particular efforts have been focused on the analysis of the structure by means of FTIR, DSC and XRD.

EXPERIMENTAL SECTION

Materials

Triphosgene, α -pinene, trifluoroacetic acid (TFA), HBr/acetic acid, were purchased from Sigma-Aldrich and L-alanine from Bachem. Anhydrous DMF, 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. Hexafluoroisopropanol (HFIP) was supplied by Apollo Scientific (UK). ω -Pentadecalactone (PDL) was purchased from Sigma-Aldrich and freshly distilled under vacuum before use. Globalide (GI) was kindly provided by Grupo Indukern (Barcelona). Novozyme 435 (*Candida antarctica* Lipase B immobilized on cross-linked polyacrylate beads) was a gift of Novozymes (Denmark), and

it was dried over molecular sieves under vacuum. 2-(Boc-amino)ethanethiol (BAET) was purchased from Sigma-Aldrich and used without further purification.

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 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. FTIR measurements were performed on a Perkin-Elmer Frontier FT-IR spectrophotometer. Spectra were obtained from powder samples using a Universal ATR sampling accessory. For the spectra recorded a variable temperature, solutions of 5 mg of polymer dissolved in 1 mL HFIP were prepared. Then, these solutions were deposited over a NaCl plate, and a film was formed after HFIP evaporation. The plate was set on Variable Temperature FTIR CELL. 8 scans were collected with a resolution of 4 cm^{-1} in the spectral region of $4000\text{--}450\text{ cm}^{-1}$. Molecular weight analysis was performed by GPC on a Waters equipment provided with RI and UV detectors. 100 μL of 0.1% (w/v) sample solution in HFIP was injected and chromatographed with a flow of $0.4\text{ mL}\cdot\text{min}^{-1}$. HR5E and HR2 Waters linear Styragel columns (7.8 mm x 300 mm, pore size $10^3\text{--}10^4\text{ \AA}$) packed with crosslinked polystyrene and protected with a pre-column were used. Molar mass averages and distributions were calculated against PMMA standards.

Thermogravimetric analysis was performed on a Mettler-Toledo TGA/DSC 1 Star System under a nitrogen flow of $20\text{ mL}\cdot\text{min}^{-1}$ at a heating rate of $10\text{ }^\circ\text{C}\cdot\text{min}^{-1}$ and within a temperature range of 30 to 600 °C. The thermal behavior was examined by differential scanning calorimetry (DSC), using a Perkin-Elmer Pyris DSC8000 apparatus. The

thermograms were obtained 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. Real time X-ray diffraction studies were carried out using synchrotron radiation at the BL11 beamline (non-crystalline diffraction, NCD), at ALBA (Cerdanyola del Vallés, Barcelona, Spain). WAXS and SAXS spectra were recorded simultaneously from powder samples sandwiched in aluminum foil and subjected to heating-cooling cycles at a rate of 10 °C·min⁻¹. The energy of the applied radiation corresponded to a 0.10 nm wavelength and spectra were calibrated with silver behenate (AgBh) and Cr₂O₃.

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. The non-invasive back scatter optic arrangement was used to collect the light scattered by the particles at an angle of 173°. The samples were analyzed in disposable cuvettes at 25 °C.

Synthesis

L-Alanine *N*-carboxyanhydride (Ala-NCA) was prepared using literature procedures.^[40,41] Crude Ala-NCA was recrystallized twice and subsequently washed with heptane, dried under vacuum and stored in a refrigerator at -20 °C until use. The ¹H NMR spectrum of this compound is given in Figure S1 of the Supporting Information (SI) file associated to this paper. PPDL₁₀ and PGI₂₀ were prepared following literature procedures.^[42,43] The synthesis of PAla₅₀ was accomplished by ROP of L-Ala-NCA initiated by hexylamine as reported elsewhere.^[40] ¹H NMR spectra of these compounds are provided in Figures S2 and S3 in the SI file.

The amino-functionalized macroinitiators, namely PPDL₁₀NH₂ and P[GI₈-co-(GINH₂)₁₂] were prepared by enzymatic ROP of their respective macrolactones and modification of the unsaturated poly(macrolactone)s by click reaction with BAET followed

by deprotection of the Boc-amino group. Experimental details of these syntheses have been previously reported by us.^[26,27] The ^1H NMR spectra of the two macroinitiators are given in Figure S4 of the SI file. *PPDL*₁₀*NH*₂: ^1H NMR (300 MHz, CDCl_3): δ = (ppm): 4.06 (t, COOCH_2), 3.65 (t, CH_2OH), 3.38 (q, CONHCH_2), 2.90 (t, CH_2NH_2), 2.63, 2.56 (2t, CH_2SCH_2), 2.30 (t, CH_2COO), 2.17 (t, CH_2CON), 1.81 (q, $\text{CH}_2\text{CH}_2\text{CH}_2\text{S}$), 1.62 (p, OCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{CO}$), 1.26 (m, $^{\text{int}}\text{CH}_2$). *P[Gl₈-co-(GlnH₂)₁₂]*: ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ = (ppm): 7.86 (bs, NH_2), 5.85 (m, $\text{CH}=\text{CH}$), 4.10-4.25 (2 m, COOCH_2), 2.95 (m, CH_2NH_2), 2.75 (m, SCH_2), 2.30 (m, CH_2CO), 1.80 (m, OCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{CO}$), 1.29 (m, $^{\text{int}}\text{CH}_2$).

Poly(ω -pentadecalactone)-*block*-poly(L-alanine) copolymers. *P(PDL*₁₀*-b-PAIa_y)*. By varying the Ala-NCA/*PPDL*₁₀*NH*₂ feed ratios, (30/1, 60/1 and 200/1), diblock copolymers with different polypeptide chain lengths were obtained. As an example for the synthesis of *PPDL*₁₀*-b-PAIa*₆₀, Ala-NCA (100 mg, 0.87 mmol) was dissolved in dried CHCl_3 (6 mL) in a Schlenk tube and placed in a 0 °C water-NaCl bath. A solution of *PPDL*₁₀*-NH*₂ (42 mg, 0.014 mmol) dissolved in dried CHCl_3 (3 mL) under nitrogen atmosphere was injected into the Ala-NCA solution through a rubber septum with a syringe. The reaction was left under stirring until the Ala-NCA had been completely consumed as monitored by FTIR spectroscopy. After full monomer conversion the polymer was precipitated into an excess of diethyl ether, recovered by centrifugation and dried under vacuum. Yield: 80%. ^1H and ^{13}C NMR spectra of a selection of these copolymers are provided in Figure S5 of the SI file. *PPDL*₁₀*-b-PAIa*₃₀: ^1H NMR (300 MHz, CDCl_3/TFA): δ = (ppm): 7.87 (bs, NH), 4.40 (bs, CH), 4.15 (t, COOCH_2), 3.85 (t, CH_2OH), 3.55 (m, NCH_2), 2.35 (t, COCH_2), 1.75 (m, OCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{CO}$), 1.59 (m, CH_3), 1.35 (m, $^{\text{nt}}\text{CH}_2$).

Poly[globalide₂₀-graft-(L-alanine)_z] copolymers. P[GI₂₀-g-(Ala)_z]. Two copolymers were prepared by using Ala-NCA/P[GI₈-co-(GINH₂)₁₂] feed ratios of 60/1 and 300/1, *i.e.* Ala-NCA/GINH₂ ratios of 5/1 and 25/1. Ala-NCA [(60 mg, 0.52 mmol) or (300 mg, 3.13 mmol)] was dissolved in 6 mL of anhydrous DMF in a Schlenk tube and placed in a 0 °C water-NaCl bath. Solutions of P[GI₈-co-(GI-NH₂)₁₂] in 2 mL of anhydrous DMF [(30 mg, 0.095 mmol) or (40 mg, 0.126 mmol) per repeating unit] were injected into the solution through a rubber septum with a syringe. The reactions were left under stirring for 5 days under vacuum, and then the polymer was precipitated into an excess of cold diethyl ether, recovered by centrifugation and dried under vacuum. Yield: 80%. The ¹H NMR spectra of P[GI₂₀-g-(Ala)₂₀] graft copolymer is provided in Figure S6 of the SI file. *P[GI₂₀-g-(Ala)₅]:* ¹H NMR (300 MHz, CDCl₃/TFA): δ = (ppm): 7.90-7.30 (m, NH), 5.50 (m, CH=CH), 4.70-4.30 (2 bs, CH), 4.20 (t, COOCH₂), 3.50 (m, NCH₂), 2.70 (m, SCH₂) 2.40 (t, COCH₂), 1.85 (m, OCH₂CH₂, CH₂CH₂CO), 1.55 (m, CH₃), 1.35 (m, ⁿtCH₂).

Nanoparticles preparation

NPs were prepared using the emulsion-solvent evaporation technique as reported elsewhere.^[26,44] Specifically, 10 mg of PPDL₁₀-*b*-PAla₃₀ diblock copolymer were dissolved in 2 mL of CHCl₃/TFA (95/5 v/v) and this solution was added to 10 mL of 1 wt-% poly(vinyl alcohol) (PVA, *M_w*~3000 g mol⁻¹) aqueous solution. The mixture was sonicated for 15 s three times to form an emulsion that was immediately poured into 10 mL of 0.3 wt-% PVA aqueous solution and magnetically stirred in an open beaker at room temperature for 3 h. After complete evaporation of CHCl₃, the produced NPs were isolated by centrifugation (11,000 rpm) and washed with de-ionized water three times prior characterization. The nanoprecipitation method was applied to drive the self-assembly of P[GI₂₀-g-(Ala)_z] graft copolymers. Briefly, 10 mg of copolymer were dissolved in 3 mL of DMF and then 3 mL of

deionized water were added dropwise under magnetic stirring (300 rpm). The mixture was dialyzed 24 h against water (MWCO 2 kDa) to remove DMF.

RESULTS AND DISCUSSION

Copolymer synthesis

The synthesis of diblock and graft copolymers based on 16-membered macrolactones, either oxacyclohexadecan-2-one (15-pentadecalactone, PDL) or oxacyclohexadecen-2-one (globalide, GI), and the α -amino acid L-alanine (L-Ala) was carried out by the two-stages sequential ring opening polymerization (ROP) process previously used by us for the synthesis of analog macrolactone-based copolymers containing L-glutamic acid or L-lysine.^[26,27] A detailed description of this chemical route with indication of all involved reagents and reaction conditions is depicted in Scheme 1.

The first stage of the synthesis was devoted to the enzymatic ROP of the macrolactone and ended with the preparation of the amino-functionalized poly(macrolactone) that was used in the second stage for initiating the ROP of the L-Ala-NCA. An amino-capped poly(pentadecalactone) PPDL₁₀NH₂ with average polymerization degree of ten was the macroinitiator used for the synthesis of the diblock copolymers

Scheme 1. Parallel routes followed for the synthesis of copolymers made of 16-membered macrolactones and L-alanine. Left: Block copolymers from pentadecalactone (PDL). Right: Graft copolymers from globalide (GI).

PPDL₁₀-*b*-PALa_y. Conversely, the unsaturated polyglobalide (PGI obtained by enzymatic ROP of GI with average polymerization degree of 20) was amino multifunctionalized by click reaction with 2-(Boc-amino)ethanethiol (BAET) and subsequent removal of the Boc group. The resulting random copolymer P[GI₈-co-(GINH₂)₁₂] containing around 60% of amino-functionalized GI-units was the macroinitiator used in the second stage for the “grafting-from” synthesis of the P[GI₂₀-*g*-(Ala)_z] copolymers. ¹H NMR spectra of the two macroinitiators are available in Figure S4 of the SI file. The ROP of Ala-NCA was triggered by the nucleophilic attack of the amino group of the macroinitiator onto the NCA carbamate carbonyl group. Different ML/Ala-NCA ratios were used for copolymerization in order to obtain a variety of copolymers with significant different Ala-segment lengths that allows a rational comparison regarding structure and properties. Synthesis results and

compositions as well as molecular weight data of all the prepared copolymers are collected in Table 1.

Table 1. Synthesis results obtained for PPDL₁₀-*b*-PAla_y and P[Gl₂₀-*g*-(Ala)_z] copolymers.

Copolymer ^a	Feed ratio ^b ML-NH ₂ /NCA	Copolymer ratio ^c ML/Ala	Yield (%)	M_n^d (g·mol ⁻¹)	M_n^e (g·mol ⁻¹)	$\bar{D}^e$	$L_{PDL,GI}/L_{Ala}^f$ (nm)/(nm)
PPDL ₁₀ - <i>b</i> -PAla ₃₀	1/30	26/74	80	4500	7300	2.1	19/11
PPDL ₁₀ - <i>b</i> -PAla ₆₀	1/60	15/85	80	6700	10200	2.9	19/22
PPDL ₁₀ - <i>b</i> -PAla ₂₀₀	1/200	5/95	70	16,600	14,000	1.4	19/73
P[Gl ₂₀ - <i>g</i> -(Ala) ₅]	1/5	25/75	80	9900	9200	4.3	39/2
P[Gl ₂₀ - <i>g</i> -(Ala) ₂₀]	1/25	8/92	60	23,500	19,700	3.9	39/8

^aCopolymer composition (in mole) expressed by subscripts are those given by NMR and rounded to the nearest five units. The subscript (y or z) for Ala refers to block and side chain average number of units for block and graft copolymers, respectively.

^bMolar ratio of amino-functionalized macrolactone unit (ML-NH₂, ML being PDL or Gl) to L-Ala NCA used in the feed for NCA ROP.

^cCopolymer composition (%-mole) determined by ¹H NMR.

^dNumber-average molecular weight determined by ¹H NMR.

^eNumber-average molecular weight and dispersity determined by GPC against PMMA standards.

^fAverage lengths of the PDL and Ala segments in the copolymers estimated on the basis of a fully extended conformation with an average projected bond length of 0.120 nm.

All the copolymerization products were analyzed by GPC using HFIP as solvent. As it is shown in Figure 1, monomodal chromatograms were obtained which allow discarding the presence of homo-oligopeptides generated by uncontrolled initiation caused by other nucleophiles that could be present in the reaction medium as it is water. The NMR analysis of the copolymers in TFA ascertained the hybrid constitution of the copolymers and afforded their molar composition in ML and Ala units which allowed appraising the coupling efficiency of the ROP reaction. For this purpose, the areas of the alpha proton arising from the alanine moiety at 4.3 ppm and the ML CH₂CH₂O signal of either PDL or Gl at 4.2 ppm were compared. Differences in composition between the block copolymer and their feeds were in all cases less than 5% indicating that an almost complete incorporation of the α-amino acid was attained by block copolymerization. In the case of the P[Gl₂₀-*g*-(Ala)₅], copolymer and feed compositions were the same but strikingly about 20% of the fed amino

Figure 1. GPC traces of the PPDL₁₀-*b*-PAla_y diblock (a), and P[Gl₂₀-*g*-(Ala)_z] graft copolymers.

acid was lost in the grafting ROP when the ML-NH₂/Ala-NCA ratio was 1/25. Nevertheless the fact that Ala grafting took place on each amino-functionalized Gl unit demonstrated that the efficiency of the initiation reaction in this case is also high, and led to infer that chain growth must be the process that becomes hindered for high Ala-NCA conversions. ¹H NMR spectra illustrative of each copolymer type are reproduced in Figure 2 with full assignment of all observed signals, and complementary ¹H and ¹³C NMR spectra are available in Figures S5 and S6 of the SI file.

Thermal properties

The thermal properties of the PPDL₁₀-*b*-PAla_y diblock and P[Gl₂₀-*g*-(Ala)_z] graft copolymers were evaluated by TGA and DSC and compared with those of their parent homopolymers PPDL₁₀ or PGL₂₀ and PAla₅₀ taken as references. All the decomposition and transition temperatures detected in these assays are listed in Table 2. Representative thermograms illustrating the thermal behavior are shown in Figures 3 and 4 and this information is complemented with additional thermograms provided in Figures S7, S8 and S9 of the SI file.

Figure 2. ^1H NMR (CDCl_3/TFA) spectra of $\text{PPDL}_{10}\text{-}b\text{-PAla}_{30}$ diblock (a), and $\text{P}[\text{Gl}_{20}\text{-}g\text{-(Ala)}_5]$ graft copolymers (b). a^i and b^L correspond to the first and last repeating unit, respectively.

Table 2. Thermal properties of the PPDL₁₀-*b*-PAla_y and P[Gl₂₀-*g*-(Ala)_z] copolymers.

Polymer	TGA ^a			DSC ^b					
	^o T _d °C	^{max} T _d °C	R _w %	1 st heating		cooling		2 nd heating	
				¹ T _m °C	¹ ΔH J·g ⁻¹	T _c °C	ΔH J·g ⁻¹	² T _m °C	² ΔH J·g ⁻¹
PPDL ₁₀	285	425	1	90	148	76	-115	91	134
PPDL ₁₀ - <i>b</i> -Ala ₃₀	210	415	11	79, 90	54	78	-19	89	24
PPDL ₁₀ - <i>b</i> -Ala ₆₀	255	328	18	75, 91	34	67	-9	90	2
PPDL ₁₀ - <i>b</i> -Ala ₂₀₀	267	340	21	73, 91	10	-	-	-	-
PGl ₂₀	370	420	2	54	84	29	-48	49	50
Poly[Gl ₂₀ - <i>g</i> -(Ala) ₅]	228	355	9	-	-	-	-	-	-
Poly[Gl ₂₀ - <i>g</i> -(Ala) ₂₀]	190	380	7	-	-	-	-	-	-
PAla ₅₀	304	375	8	-	-	-	-	-	-

^aOnset for 5% weight loss (^oT_d) and maximum rate (^{max}T_d) thermal decomposition temperatures measured in the TGA analysis performed under inert atmosphere. R_w: weight (%) remaining after heating at 600 °C.

^bMelting (T_m and ΔH_m) and crystallization (T_c and ΔH_c) temperatures and enthalpies measured by DSC.

The TGA analysis of PPDL₁₀ and PGI₂₀ evidenced the high thermal resistance inherent to these polyesters. As it is seen in Figure 3, they decompose through a rather simple process that does not begin to be clearly perceivable up to above 400 °C. PAla₅₀ is also fairly stable to heat displaying a single decomposition temperature with maximum rate at 375 °C. The thermal decomposition traces recorded for the copolyesters are in general more complex and show significant weight losses at temperatures intermediate of those registered for their corresponding homopolymers. The derivative curves of these traces (Figure S7) contain 3-4 peaks corresponding to other so many decomposition steps (only the ^{max}T_d for the main peak has been listed in Table 2). The onset temperatures of the copolyesters (^oT_d measured for 5% of weight loss) were markedly lower than those of the poly(macrolactone)s and they tend to diminish with copolymerization to become even lower than that of PAla₅₀. The TGA of physical mixtures of PAla₅₀ with either PPDL₁₀ or PGI₂₀ indicated no substantial changes in the onset decomposition temperatures

compared to the individual homopolymers (Figure S8). It may be concluded therefore that copolymerization entails a noticeable decreasing in thermal stability probably due to the end-group effect associated to the presence of short PAla segments. Nevertheless, the onset temperatures values should be taken with caution since the presence of minor amounts of water, which is difficult to remove, may alter significantly their determination.

Figure 3. TGA traces of PPDL₁₀-*b*-PAla_y and P[Gl₂₀-*g*-(Ala)_z] copolymers compared to those of their parent homopolymers.

The occurrence of thermal transitions taking place in the copolymers was examined by DSC of samples subjected to heating/cooling/heating cycles within the 25-170 °C range. The cycle traces recorded for PPDL₁₀-*b*-PLAla₃₀ diblock and P[(Gl₂₀-*g*-(Ala)₅] graft copolymers, which may be taken as representatives, as well as those recorded for their parent homopolymers are depicted in Figure 4. The DSC traces obtained for the other copolymers synthesized in this work have been added to the SI file (Figure S9). The traces registered for PPDL₁₀ and PGI₂₀ were characteristic of semicrystalline polymers showing ¹T_m's at 90 °C and 54 at the first heating °C, and ¹T_c's at 76 °C and 29 °C, upon cooling, respectively. The recrystallized samples displayed melting peaks comparable to

those observed for the pristine samples with small deviations in temperatures and enthalpies. These results are in full agreement with data published on the high molecular weight PPDL^[45] and PGI homologues.^[43] On the other hand, the thermogram produced by a sample of PAla₅₀ coming directly from synthesis showed a broad endotherm with its maximum at 70-80 °C that was attributed to solvent volatilization since it almost fully disappeared when the sample was previously dried at 45 °C for several hours under vacuum. No other heat exchange was detected for PAla₅₀, neither at heating nor at cooling through the analyzed range of temperatures. This is in agreement with that has been reported for the thermal behavior of PAla in the solid state which is characterized by the occurrence of a phase transition from the α -form to the β -form taking place at temperatures above 300 °C. Melting of PAla cannot be experimentally observed because it would take place at temperatures above decomposition.

The DSC pattern characteristic of PPDL₁₀-*b*-PAla_y copolymers may be represented by that recorded for PPDL₁₀-*b*-PAla₃₀ which is shown in Figure 4a. The first heating trace taken from the copolymer (previously dried as it was indicated for PAla₅₀) showed two endothermic peaks at 77 °C and 90 °C with similar intensities which are attributed to the respective melting of two copolymer populations differing in crystallite size. At cooling from 170 °C, a crystallization peak was observed at 78 °C, and at the second heating, the initial melting peak appearing at 90 °C was recovered accompanied of a broad weak endotherm that spread over a 70-80 °C range. These results doubtlessly indicate that the PPDL block in this copolymer is crystallized and that it can be reversibly melted and recrystallized. Similar results were observed for the other two block copolymers with the only difference that no recrystallization was observed for PPDL₁₀-*b*-PAla₂₀₀ which must be interpreted as due to the high diluent effect exerted by the long PAla block present in this copolymer (Figure S9). The DSC results obtained for the P[GI₂₀-*g*-(Ala)_z] graft copolymers differed noticeably from those described for PPDL₁₀-*b*-PAla_y block copolymers in spite of that the

melting-crystallization behavior displayed by PGI₂₀ and PPDL₁₀ was very similar. The heating-cooling traces registered for P[GI₂₀-*g*-(Ala)₅] (Figure 4b) did not display any sign of crystallinity indicating that PGI₂₀ became unable to crystallize after grafting. The same result was obtained in the analysis of P[GI₂₀-*g*-(Ala)₂₀] (Figure S9). It can be therefore concluded that, as expected, the graft copolymers are essentially amorphous whereas the block copolymers are able to show crystallinity for whichever composition.

Figure 4. DSC traces recorded for a) PPDL₁₀-*b*-PLA₃₀ diblock and b) P[GI₂₀-*g*-(Ala)₅] graft copolymers and for their parent homopolymers.

The influence of the PALa_y block on the crystallizability of the PPDL₁₀ block in PPDL₁₀-*b*-PALa_y copolymers was examined by measuring the increment of the relative crystallinity produced in their isothermal crystallization as a function of time. Results obtained for the three block copolymers studied in this work as well as for the poly(pentadecalactone) homopolymer are plotted in Figure 5, where it is clearly shown that the presence of the PALa_y block whatever its length enhanced the crystallization rate of the PPDL₁₀ block. Crystallization parameters obtained by the Avrami kinetic analysis of the four polymers are compared in Table S1 included in the SI file. Half-crystallization times of 2.7-2.9 min were found for the copolymers whereas a value of more than 6 min was found for PPDL. Such a crystallization rate enhancing effect seems to be however something

more intense for shorter PAla_y blocks, a fact that may be due to the high dilution produced by the PAla_{200} block. This result is in line with the non-observance of recrystallization for the $\text{PPDL}_{10}\text{-}b\text{-PAla}_{200}$ copolymer when it was cooled at constant rate from the melt to room temperature (Figure S9).

Figure 5. Evolution of the relative crystallinity as a function of time in the isothermal crystallization at 82 °C of the $\text{PPDL}_{10}\text{-}b\text{-PAla}_y$ diblock copolymers.

Chain conformation and structure

The crystal structure of poly(macrolactone)s has been reported by different authors. PPDL is known to adopt a pseudo-rhombic monoclinic structure with a unit cell of approximate dimensions $a = 0.75$ nm, $b = 0.5$ nm, and $c = 2.0$ nm and $\alpha = 90.0^\circ$.^[42,46] The X-ray diffraction spacings characteristic of this structure are those at 0.40 nm and 0.37 nm corresponding to 110 and 200 interplanar distances. On the contrary, the crystal structure of PGI has not yet been resolved but there indications that it must not be significantly dissimilar to that of PPDL.^[25] On the other hand, the polypeptide PAla_n is known to be usually arranged in one of the two ordered structures commonly found in polypeptides, *i.e.* the α -helical or the β -sheet forms, with preference for one or the other depending on both

chain length and crystallization conditions. The helical structure consists of a hexagonal arrangement of 13/5 helices that is characterized by a strong reflection at about 0.75 nm. The β -form is a layered structure made of nearly fully extended chains with characteristic X-ray reflections at 0.53 nm and 0.45 nm.^[47] FTIR spectra are usually taken to support the chain arrangement adopted by polypeptides. The absorption bands at 1650 cm^{-1} (Amide I) and 1545 cm^{-1} (Amide II) are taken as characteristic of the α -helix whereas bands at 1630 (Amide I), 1535 (Amide II) and 700 cm^{-1} (Amide V) are used for identifying the β -form. The helical form is known to be favored by high molecular weights and temperatures. It has been also reported that the helical form is irreversibly converted into the layered form by heating at temperatures close to 300 °C, a transition that may be followed by both XRD and FTIR.^[47]

The arrangements adopted by the block and graft PML-PAla copolymers in the solid state and the changes that they may undertake by effect of heating were examined by FTIR and XRD at variable temperature. Firstly the FTIR study was carried out using powder samples in order to get insight into the secondary structure adopted by the polypeptide chains. The 1500-1800 cm^{-1} region of the FTIR spectra, in which Amide I and Amide II bands appear, is displayed in Figure 6 for PPDL₁₀-*b*-PAla_y diblock and P[Gl₂₀-*g*-(Ala)_z] graft copolymers as well as for the homopolypeptide PAla₅₀. Just to note that the band due to the polyester C=O stretching is observed in this region at 1730 cm^{-1} with an intensity that correlates well with the relative content of ester groups in the copolymers. The spectrum of PAla₅₀ shows a main absorption peak at 1650 cm^{-1} with a prominent shoulder at 1630 cm^{-1} that correspond to the Amide I band of the helical and sheet forms, respectively. Accordingly, the Amide II band was found to consist of a broad absorption with a maximum at 1540 cm^{-1} and a poorly visible shoulder at around 1520 cm^{-1} . The spectra recorded from the copolymers displayed a pattern similar to that of PAla₅₀ but differing in the relative intensity of the Amide I and II bands arisen from each of the two

Figure 6. Amide I and II region of FTIR spectra of a) PPDL₁₀-*b*-PAla_y diblock and b) P[Gl₂₀-*g*-(Ala)_z] graft copolymers.

forms. In the case of the graft copolymers (Figure 6b) the amounts of polypeptide arranged in helical and extended conformation are comparable for a side chain length of 20 Ala

units whereas the latter is the only almost conformation present when the length of the Ala segment reduced to 5 units. Strikingly the behavior observed for the block copolymers is just the opposite. In this case it is the α -form that is largely predominant for shorter polypeptide lengths although it is true also that the β -form seem to be minority along the whole series. It may be then speculated that the polyester in the crystallized state must exert certain influence on the molecular arrangement adopted by the polypeptide with the result of an inversion in its expected conformational preferences. The response given by FTIR spectra to heating is in line with these observations. The PPDL₁₀-*b*-PAla₂₀₀ copolymer containing similar amounts of α and β -form at room temperature became entirely arranged in the α -form when heated at temperatures above 60 °C which is close to the melting temperature of the polyester block. On the contrary, the α/β composition of P[Gl₂₀-*g*-(Ala)₂₀], in which the polyester counterpart is not ordered, appears to be invariable with temperature. Plots of the FTIR spectra of PPDL₁₀-*b*-PAla₂₀₀ and P[Gl₂₀-*g*-(Ala)₂₀], at temperatures increasing from 25 °C to 250 °C are shown in Figure S10 of the SI file.

The X-ray diffraction study was performed on powder samples using synchrotron radiation at variable temperature and recording the scattered light at real time. WAXS and SAXS data were simultaneously registered and the changes taking place by effect of heating or cooling were visualized by comparative representation of the scattering profiles as a function of temperature (Figure 7 and Figures S11-S13 in the SI file).

Figure 7. Evolution of the WAXS (a and b) and SAXS (a' and b') diffraction profiles of $\text{PPDL}_{10}\text{-}b\text{-PAla}_{30}$ diblock and $\text{P[Gl}_{20}\text{-}g\text{-(Ala)}_{20}\text{]}$ graft copolymers recorded at increasing temperature from 10 °C to 200 °C.

The WAXS profile registered at 10 °C from $\text{PPDL}_{10}\text{-}b\text{-PAla}_{30}$ (Figure 7a) clearly showed the two characteristic peaks of PPDL at 0.41 nm and 0.37 nm. At heating these peaks kept unaltered until temperature reached around 90 °C where they completely disappeared. According to DSC data such behavior corresponds to the melting of the crystalline PPDL block. In fact, both peaks were almost completely recovered upon cooling at temperatures around 60 °C. A reflection at 0.76 nm with a moderate intensity that slightly increased with temperature was also detected in the WAXS profiles. This reflection

is made to correspond to the hexagonal packing of PAla₃₀ α -helices. Additionally a careful inspection of the low temperature profile revealed the presence of low intensity peaks at 0.49 nm and 0.45 nm that stayed invariable at 200 °C, and which could be attributed to the presence of some amount of polyalanine in the β -form. The SAXS profile registered for PPDL₁₀-*b*-PAla₃₀ (Figure 7a') at 10 °C displayed weak reflections at 20, 10 and 5 nm that remained unaltered up to temperatures near to that of PPDL melting was reached. It seems therefore that a 1D structure with a main spacing of 20 nm (second and fourth orders at 10 nm and 5 nm) and consisting of a biphasic alternating arrangement of the two blocks, is adopted by this copolymer. Since all SAXS signals disappeared upon heating at temperatures close to 90 °C, it can be inferred that the nanometric structure becomes unstable as soon as the polyester phase is molten.

The X-ray diffraction results produced by the P[Gl₂₀-*g*-(Ala)₂₀] graft copolymer were largely dissimilar. As it is seen in Figure 7b, no peaks characteristic of polyester were observed in these profiles so that all the discrete reflections there present have to be interpreted to arise from the polypeptidic phase. Peaks at 0.49 nm and 0.45 nm characteristic of β -form and 0.76 nm of α -form observed at low temperature remained almost invariable at heating up to 200 °C. This behavior is in agreement with that observed by FTIR and confirms the thermal stability of the two forms in the graft copolymers over the studied range of temperatures. The SAXS produced by P[Gl₂₀-*g*-(Ala)₂₀] and how it changed with temperature is shown in Figure 7b'. No scattering was observed below ~150 °C indicating the absence of any order or discrete phase segregation in this copolymer at low temperature. At high temperature, *i.e.* above 160 °C, a broad signal corresponding to a spacing of approximately 6.6 nm emerged and became more pronounced as temperature increased. Unfortunately, there are not enough data available nor accessible precedents that allow conjecturing with a minimum detail on the nature of the arrangement responsible for such scattering.

Copolymer self-assembly in aqueous environment

The copolymers studied in this work cannot be actually classified as amphiphilic since the PML block is highly hydrophobic and PAla cannot be considered a hydrophilic polypeptide although its affinity for water is greater than that of the polyester. In fact, PAla segments have been combined with other segments made of charged α -amino acids or water-soluble monomers in order to generate well-defined amphiphilic block or graft copolymers able to display a strong tendency to phase separation.^[28-32]

Nevertheless, it was interesting to explore in a comparative manner the spontaneous assembling behavior that could display PPDL₁₀-*b*-PAla_y block and P[Gl₂₀-*g*-(Ala)_z] graft copolymers when they are freely placed in an aqueous environment. Both the emulsion-evaporation solvent and the nanoprecipitation techniques were used to drive the self-assembly of a selection of PML-PAla copolymers in recognizable nano-objects and the obtained results are given in Table 3. Both PPDL₁₀-*b*-PAla₃₀ and PPDL₁₀-*b*-PAla₂₀₀ became self-assembled in nanoparticles with a low positive zeta-potential and diameters about 300 nm and 350 nm, respectively. The DLS profiles and SEM images of these nanoparticles are shown in Figure 8. On the other hand, neither the emulsion-solvent evaporation nor nanoprecipitation method induced the self-assembly of P[Gl₂₀-*g*-(Ala)₅] copolymer so that only unspecific aggregates of large sizes were observed instead. The DLS profile recorded from this sample is available in Figure S14 of the SI.

Table 3. Characterization of nanoparticles by DLS.

Polymer	<i>D</i> (nm)	<i>DPI</i>	ζ (mV)	Used method
PPPL ₁₀ - <i>b</i> -PLAAla ₃₀	300	0.29	6.3	emulsion-evaporation
PPPL ₁₀ - <i>b</i> -PLAAla ₂₀₀	350	0.39	7.5	emulsion-evaporation
P[Gl ₂₀ - <i>g</i> -(Ala) ₅]	60/3000	0.88	-	nanoprecipitation
P[Gl ₂₀ - <i>g</i> -(Ala) ₂₀]	n.o.	n.o.	-	nanoprecipitation

n.o. = not observed.

Figure 8. DLS profiles and SEM images of PPDL₁₀-*b*-PAla₃₀ (a) and PPDL₁₀-*b*-PAla₂₀₀ (b) diblock copolymers.

An issue of relevance to the potential application of these nanoparticles in the drug delivery field is their degradability in aqueous medium by the copolymer hydrolysis. Degradation assays carried out by Heise et al.^[25] with both PPDL and PGI showed that these two poly(macrolactone)s remained essentially unaltered after incubation for several months in PBS at 37 °C, both in the presence and in the absence of lipases. The main reason for the hydrolytic stability of these polyesters was suggested to be their high hydrophobicity, which prevents water from penetration into the materials to initiate degradation. It may be thought however that the insertion of polypeptide segments on poly(macrolactone)s could enhance the hydrolyzability of the polyester chain. In a recent work, we tested a copolymer of PGI grafted with glutamic acid by incubating it in aqueous

buffer pH 4.2 at 37 °C for several hours, and following the possible chemical modifications by NMR.^[27] No signal changes indicative of hydrolysis of the polyester or the polypeptide counterpart were detected. It can be reasonably inferred therefore that none of the poly(ML-Ala) copolymers included in the present study will display noticeable hydrolytic degradation within the time intervals in which they would be usually applied. Nevertheless a specific study covering a wide range of incubation conditions and considering the assistance of enzymes should have to be made in order to reach firm conclusions in this regard

Conclusions

The sequential ROP polymerization previously used by us for the synthesis of poly(macrolactone)-poly(α -amino acid) copolymers made of either L-Lys or L-Glu has proven to be extensible to the preparation of block and graft copolymers made of L-Ala. The polyester block in the poly(pentadecalactone)-*block*-poly(α -L-alanine) copolymers was in the crystalline state and was able to crystallize from the melt at a crystallization rate enhanced by the presence of the polypeptide block. On the contrary, the polyester main chain of the poly[globalide-*graft*-(α -L-alanine)] copolymers was invariably in the disordered state. Regarding the polypeptide counterpart, a conformational mixture of α -helical and β -forms was present in both types of copolymers but the dependence of their composition on the polypeptide length and its response to heating was found to be opposite. The conformational behavior observed in graft copolymers was in agreement with expectations but in block copolymers the α/β ratio decreased with the increasing length of the polypeptide chain and with decreasing temperature. The crystalline nature of the polyester domain and the melting-crystallization transition happening in the block copolymers could be invoked to be responsible for this anomalous behavior. Such differences in crystallinity and conformation become reflected in the different ability displayed by these copolymers

to be self-assembled. Only the block copolymers were able to form nanometric particles, a property that could be associated to the expected tendency of the polyester block to build a crystalline core with subsequent outwards segregation of the poly(α -amino acid) segment arranged in the α -helical form. The incapacity of the graft copolymers to form nanoparticles may be attributed either to a defective separation of the poly(α -amino acid) and polyester domains or to an extensive primary particle aggregation caused by hydrogen-bonding interaction between the peripheral polypeptide arranged in the β -form.

Associated information

^1H NMR of Ala-NCA, PPDL₁₀ and PGI₂₀ and macroinitiators. ^1H and ^{13}C NMR spectra of selected copolymers. TGA derivative curves and DSC traces of different copolymers and their mixtures. FTIR spectra of copolymers recorded at increasing temperatures. Evolution of WAXS and SAXS profiles of different PPDL₁₀-*b*-PAla_y and P[GI₂₀-*g*-(Ala)_z] copolymers registered at heating and cooling. DLS for the P[GI₂₀-*g*-(Ala)₂₀] nanoparticles.

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Graphical Abstract

PPDL-*b*-PAla diblock and P(GI-*g*-Ala) graft copolymers were synthesized by ROP of L-alanine NCA initiated by amino-functionalized polypentadecalactone and polyglobalide. PPDL-*b*-PAla have the PPDL phase crystallized and the PAla in α -helical conformation. P(GI-*g*-Ala) have the polyester main chain in disordered state and the PAla side chains in the β -sheet form. Nanoparticles could be prepared only from block copolymers.