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Research Article

Synthesis of ^{3}H and fluorescence-labelled poly (DL-Lactic acid)

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Summary

The novel polymer modification method based on the activation of an aliphatic polyester chain by using lithium diisopropylamide at low temperature to form a polycarbanion bearing nucleophilic sites in a position α to the carbonyl groups was applied to *rac*-poly (lactic acid), PLA_{50}, to label this polymer with radioactive tritium atoms or with a fluorescent dye. Despite simultaneous partial chain degradation, radioactive PLA_{50} and fluorescent PLA_{50} with $\overline{M_n} = 14\,000\ g\,mol^{-1}$, $\overline{M_w}/\overline{M_n} = 1.9$ and $\overline{M_n} = 32\,000\ g\,mol^{-1}$, $\overline{M_w}/\overline{M_n} = 2.4$, respectively, were obtained. The specific activity of the final compound was $3.7\ \mu Ci\,g^{-1}$ that squared with 0.3‰ substitution reaction. The fluorescent polymer was substituted with 0.25% ratio. This work shows that the LDA activation method appears to have considerable potential. Copyright © 2001 John Wiley & Sons, Ltd.

Key Words: poly(DL-lactic acid); lithium diisopropylamide; polymer chemical modification; carbanion; nucleophilic addition; tritium-labelling; fluorescence-labelling; bioresorbable polymers; aliphatic polyesters

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Introduction

High molecular weight polymers and stereocopolymers derived from lactic acid (PLA_x, where x stands for the percentage of L-lactoyl units) are aliphatic biocompatible and degradable polyesters obtained by ring opening polymerization of lactide diastereoisomers taken in suitable proportions. Lactides can be co-polymerized with other cyclic monomers such as glycolide (GA) or ε-caprolactone (CL) to generate a great number of degradable and biodegradable aliphatic polyesters.[1] Poly (L-lactic acid) (PLA_{100}) and several co-polymers with glycolide (PLA_xGA_y, where y stands for the percentage of glycoloyl units) are currently used in human bone surgery in the form of bone plates, screws, pins, (Bioscrew®, Phusiline®, Sysorb®, etc.)[2] and in wound treatment as suture materials or as soft tissue temporary prosthetic reinforcements (Vicryl®, Galactin910®).[3,4] Many amorphous members of the PLA_xGA_y family have been proposed as suitable matrices for controlled drug delivery, [5] some of them being commercially available (Decapeptyl® LP, Zoladex®, Enantone®, Sandostatine®, etc.).[6–8] For all these applications, monitoring the fate of degradation products *in vitro* and *in vivo* is one of the critical steps to evaluate degradation characteristics and to extend the biocompatibility criterion to degradation products. For the sake of monitoring their fate in complex living systems, degradable polymer chains have to be labelled. According to the literature, fluorescence and radioactivity-labelling are the two major means generally used by biologists and pharmacists to monitor the fate of biomolecules or drug or drug metabolites. Fluorescence labelling is generally obtained by reaction of functionalized polymers with fluorescent dyes to create potentially labile links (amide, sulphamide).[9] The structure of the resulting compounds is thus notably different from that of the parent co-polymers. Radio-labelling with foreign nuclides such as $^{125/131}I$ raises the same risk of misleading data. Therefore radio-labelling using radioactive isotopes of atoms normally present in the investigated molecules such as carbon or hydrogen must be preferred in order to preserve the intrinsic chemistry of the molecules and their physico-chemical properties as well.

^{14}C-labelling of PLA_x stereoco-polymers was reported at a very early stage by Kulkarni *et al.*[10] The authors showed that no significant radioactivity was recovered in the faeces or urine, but that the polymer is degraded and eliminated, possibly through CO_2 in the breath. [^3H]-labelled PLA of very low specific activity was also mentioned in the

literature several years ago, but the tritiation method was not described.[11] The tritiation of PLA_xGA_y was recently revisited with the aim of providing polymers labelled at higher specific activity which is necessary in order to have high sensitivity when monitoring the degradation and dispersion of degradation by-products in complex living systems. For these reasons, lactide and glycolide tritiations were achieved catalytically to generate [^{3}H]-labelled cyclic dimers.[12,13] Radioactive lactides with specific activities ranging up to 22.7–30.3 $mCi\,mg^{-1}$ were thus obtained and the percentage of recovered lactide was in the 50–60% range. A highly radioactive tritiated lactide diluted with cold lactide was polymerized using stannous octoate or Zn lactate in order to synthesize highly tritiated PLA polymers. The recovered tritiated polymeric compounds had specific activities ranging from 0.8 to 1.2 $mCi\,mg^{-1}$. Polystyrene-related weight average molecular weights $\overline{M_w}$ were in the range 25 000–60 000 $g\,mol^{-1}$, $\overline{M_w}/\overline{M_n}$ being lower than 2.5.

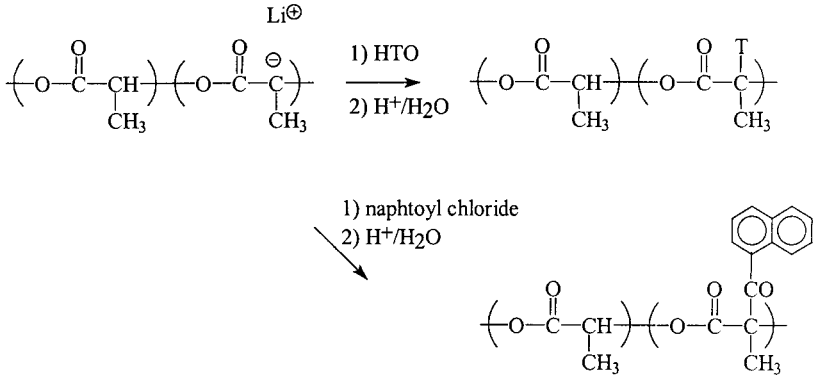
In this paper we wish to report a simpler and versatile process to prepare [^{3}H] radio-labelled and fluorescence-labelled high molecular weight PLA using the method already reported for poly(ε-caprolactone), PCL.[14,15] The labelling is based on a chemical modification carried out after activation of carbonyl-bearing polymer chains to give a polycarbanion by using lithium diisopropylamide (LDA) and subsequent coupling of either tritiated water or a fluorescent dye.

Results and discussion

Synthesis of labelled-PLA

In a previous paper we have shown for the first time that LDA can be used at low temperature to extract active protons from poly (ε-caprolactone) (PCL) polymer chains and thus generate a polyanion with carbanionic sites created at positions α to the carbonyl groups.[14] It was also shown that these carbanionic sites can react with electrophilic reagents thus allowing the grafting of functional groups onto PCL chains. Despite a simultaneous mild chain cleavage reaction that leads to a lowering of the molecular weight, the method appeared very versatile, as exemplified by the grafting of benzaldehyde, naphthoyl chloride, benzyl chloroformate and iodomethane [14] or the synthesis of radioactive tritiated [^{3}H]-PCL chain using tritiated water (HTO) as the electrophile.[15]

Given the increasing interest in lactic acid-based polymers, stereoco-polymers and co-polymers, attempts were made to apply the method to one member of the lactic acid stereoco-polymer family, namely PLA_{50} where 50 stands for the percentage in L-units within the chains according to a recommendation aimed at better reflecting the structure of the considered PLA polymer.[16,17] In a first approach, HTO and naphthoyl chloride were selected as electrophiles to synthesize radio-active- and fluorescence-labelled PLA via activated PLA chains according to Scheme 1:



Scheme 1.

The labelling results are given in Table 1. A low concentration of LDA (0.25 equivalent/monomeric unit for tritiation and 0.1 for fluorescence labelling) was used to avoid excessive side-reactions and the resulting dramatic degradation of polymeric chains observed in preliminary experiments.[14] PLA_{50} was recovered in 36% yield for the [^{3}H]-labelling and 47% yield for the fluorescence-labelling, after precipitation in methyl alcohol.

Table 1. Labelling of PLA_{50} by chemical modification via anionic way with LDA at −78°C under argon flow

Label	LDA proportion	Substitution ratio %	Yield %	$\overline{M_n}$ (refractometry)	$\overline{M_w}/\overline{M_n}$
Naphthoyl chloride	0.1	0.25	47	32000	2.4
HTO	0.25	0.03	36	14000	1.9

SEC chromatograms showed that chain lengths were affected to some extent by the reaction. Starting from a polymer having a molecular weight in the range of $\overline{M_n} = 170\,000\ \mathrm{g\,mol^{-1}}$ $\left(\overline{M_w}/\overline{M_n} = 1.5\right)$, we ended up with recovered [^{3}H]-PLA and fluorescent-PLA having $\overline{M_n} = 14\,000\ \mathrm{g\,mol^{-1}}$ $(\overline{M_w}/\overline{M_n} = 1.9)$ and $\overline{M_n} = 32\,000\ \mathrm{g\,mol^{-1}}$ $(\overline{M_w}/\overline{M_n} = 2.4)$, respectively.

Given the labels attached to the PLA chains, the refractive index detector generally used in SEC was complemented by a flow scintillation detector or a fluorimetric detector for the radioactive and the fluorescent PLAs, respectively. The corresponding SEC profiles (Figures 1 and 2) show that the two labelling methods were effective, the retention times deduced from refractive index detection being almost the same as those given by the complementary detection system. Moreover, no significant amounts of low molecular weight radioactive compounds were detected.

The specific activity of the [^{3}H]-labelled-PLA obtained was $3.7\ \mu\mathrm{Ci\,g^{-1}}$, sufficiently high for degradation studies under *in vitro* or *in vivo* conditions (45 000 times higher than the detection level of the

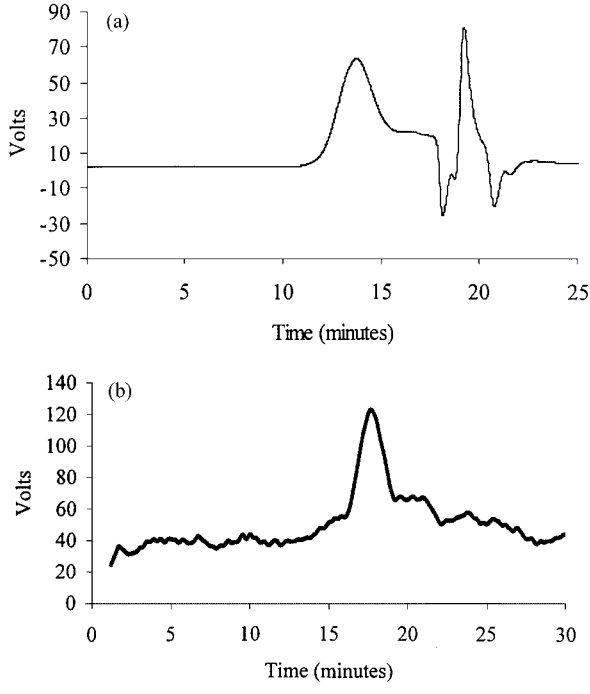


Figure 1. SEC chromatograms of tritiated-PLA in THF after refractometric detection (a) and flow scintillation detection (b)

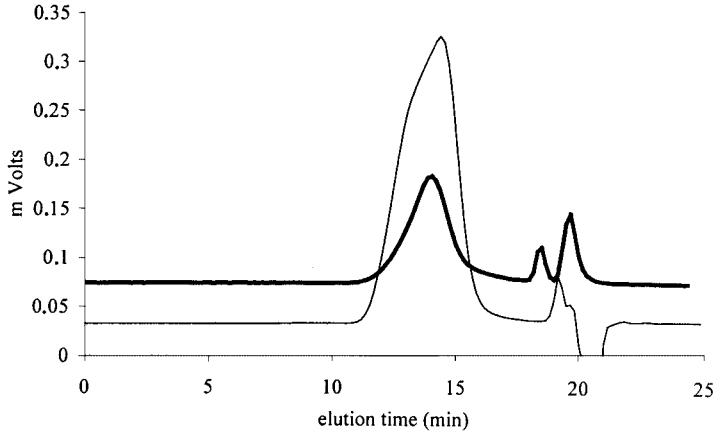


Figure 2. SEC chromatograms of fluorescent-PLA in THF after refractometric detection (—) and fluorimetric detection at $\lambda_{ex} = 298$ nm and $\lambda_{em} = 365$ nm (—)

selected scintigraphic method). Based on the specific activity of the diluted HTO and on the fact that the hydrogen and the tritium atoms of HTO could react identically, the substitution ration was evaluated at 0.3‰.

The substitution ratio of the fluorescence-labelling PLA_{50} ($\approx 0.25\%$) was determined by ^{1}H-NMR spectroscopy using the ratio of the area of the naphthoyl protons peaks between 7.3 and 9 ppm to the area of the CH_3 signal (peaks at 1.5 ppm). The binding of the fluorophore and the absence of small molecules entrapped in the polymer matrix were checked by SEC (Figure 2). In both reactions the substitution ratio was high enough to label the polyester.

Control of labelling stability

One of the main limitations to the use of radioactive materials as tracers is radiolysis. This is a general phenomenon that can lead to degradation and thus be the source of an additional route to by-products formation.

Another problem is the possible presence of soluble radioactive remnants from the synthesis stage. The scintillation-detected SEC chromatogram of the [^{3}H]-labelled-PLA immediately after recovery from purification is free of such products (Figure 1(b)). The recovered [^{3}H]-labelled-PLA polymer was considered with the sensitivity of the device initially free of low molecular weight radioactive compounds such as tritiated water or radioactive low molecular weight compounds issued from the substitution reaction.

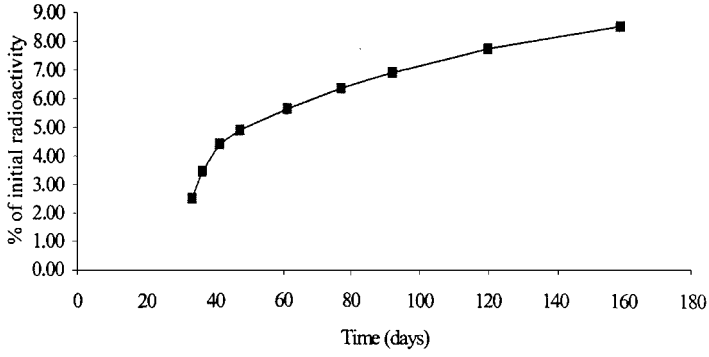


Figure 3. Plot of the cumulative loss of radioactivity versus time with reference to the initial radioactivity of the polymer

The radioactivity of the ^{3}H-labelled PLA_{50} stored at 4°C was checked over the course of 5 months (Figure 3). At the end of this time some 8% of the polymer radioactivity had been released. The nearly linear plot shows that the rate of release is fairly constant with no sign of acceleration. The sensitivity of the approach is illustrated by the fact that during the same time interval there have been no changes in the SEC of the tritiated polymers. These results are similar in many respects to those obtained previously for $^{1}H \rightarrow {}^{3}H$ catalytic exchange on solid lactide.[13] The low radiolysis rate (0.82% per month) allows a prolonged stocking of the radioactive-labelled PLA.

Experimental

Chemicals

PLA was synthesized from DL-lactide (Purac, Gorinchem, The Netherlands). The monomer was purified by crystallization at room temperature after solubilization in acetone at 50°C and filtration. The crystals were dried under vacuum after grinding. The polymer $\left(\overline{M_n} = 170\,000, \overline{M_w}/\overline{M_n} = 1.5\right)$ was obtained in 85% yield.

The LDA, salts and solvents were obtained from commercial sources and the tritiated water ($100\,mCi\,g^{-1}$, $1.8\,Ci\,mol^{-1}$) from NEN Life Science Products (Boston, USA).

THF was distilled on benzophenone/sodium until a deep blue colour was obtained.

Methods

[^{3}H]-*labelling*. A solution of LiCl (4.25 g, 0.1 mol) in 100 ml of anhydrous THF was inserted in a 500 ml three-necked round-bottomed flask, previously flame dried and kept under a flow of argon dried through three gas-drying units filled with sodium hydroxide pellets, molecular sieves and silicagel, respectively. PLA (5.76 g, 0.08 mol of monomer unit) was added and the solution was stirred until dissolution. The temperature was then lowered to -78°C using a dry ice/acetone mixture and the solution was kept under stirring. 2 M LDA in THF/*n*-heptane (10 ml, 0.02 mol : 0.25 equivalent/monomeric unit) was then introduced with a syringe through a rubber septum. The mixture was kept with stirring at -78°C for 5 min. 640 µl of HTO (0.035 mol at 100 mCi g^{-1}) in 4 ml of THF was introduced into the reaction flask through a septum and the stirred reaction solution was kept for 5 min at -78°C. An aqueous solution of ammonium chloride (10 g of NH_4Cl in 200 ml water) was then added into the reaction flask and the mixture gently stirred at room temperature. The tritiated polymer was extracted using dichloromethane (2×100 ml^2). The combined organic phases were washed with distilled water (2×100 ml^2) and dried over anhydrous $MgSO_4$. After filtering the solid hydrated salt, the solvent was partially evaporated under reduced pressure and the polymer precipitated by addition of analytical grade methyl alcohol. The immediately formed precipitate was washed with methyl alcohol until the solution was clear. The tritiated polymer was then dried under vacuum for several hours.

Fluorescence-labelling. 2 M LDA solution in THF/*n*-heptane (2 ml, 0.004 mol : 0.1 equivalent/monomeric unit) was introduced to a PLA solution consisting of 2.88 g (0.04 mol in monomeric units) in 100 ml anhydrous THF. The formation of the carbanion was allowed to proceed for 5 min at -78°C. Then naphthoyl chloride was added in excess (0.7 ml) and the mixture was allowed to react for 5 min at -78°C. The resulting fluorescent polymer was recovered as in the case of the tritiated polymer.

Radioactivity assessment. The specific activity of the tritiated PLA was determined using a Liquid Scintillation Analyzer, Tri-Carb 2100 TR Packard. Typically, 10 mg of polymer was dissolved in 1 ml of THF and 50 µl of the sample solution was diluted in 7 ml of Ultima Flo^{TM} AP, Packard, and counted.

Radiolysis assessment. The powdered tritiated polymer was stored in a refrigerator at 4°C for several months. The radioactivity was assessed at intervals. Typically 54 mg of the radioactive PLA was dissolved in 1 ml of THF. Two aliquots of 100 µl were diluted in 7 ml of Ultima Flo^{TM} AP, Packard, a solvent-based LSC-cocktail, and counted using a Tri-Carb 2100 Scintillation Counter. The rest of the THF solution was distilled under vacuum and any volatile compounds were collected in a liquid nitrogen trap. Aliquots of the distilled and collected THF were analysed for the presence of radioactivity.

^{1}H *nuclear magnetic resonance (*^{1}H *NMR)*. The substitution ratio of the fluorescent polymer was determined by ^{1}H NMR spectra recorded at room temperature using Bruker spectrometers operating at 250 MHz. Deuterated chloroform was used as solvent and chemical shifts were expressed in ppm from the tetramethylsilane (TMS) resonance.

Size exclusion chromatography (SEC) and label assessment. Molecular weights were determined by SEC using an instrument fitted with a 60 cm long column using 5 µm mixed C PLgel as the stationary phase, THF at 1 cm^{3} min^{-1} flow rate as the mobile phase, and a Waters 410 refractometric detector. A flow scintillation analyser, Radiomatic Flo-one^{TM} Beta Packard, for detection of [^{3}H]-PLA or a scanning fluorescence detector, Waters 470, for detection of fluorescent-PLA, were used on-line to evaluate the molecular weight of the labelled polymer. Typically, 10 mg of polymer was dissolved in 1 ml THF and the resulting solution filtered on a 0.45 µm Millipore filter before injection of 20 µl of the sample solution. $\overline{M_n}$ and $\overline{M_w}$ data were referred to polystyrene standards.

DSC analysis. DSC thermograms were obtained by using a DSC6 Perkin Elmer analyser under a flow of nitrogen gas at a 10°C min^{-1} heating rate.

Conclusion

Tritiated PLA and fluorescent-labelled PLA have been prepared by anionic activation using LDA at low temperature. Despite the greater sensitivity of PLA chains as compared to those of PCL, labelled PLA with molecular weight in the 20 000 g mol^{-1} range and specific activity

of 3.7 μCi g^{-1} can be easily obtained through a rapid and efficient one-pot reaction. The method is potentially extremely versatile and could be used to label other aliphatic polyesters. Hydrolytic degradation of labelled poly(acid lactic) is described in a further article.

Acknowledgements

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