

Synthesis and characterization of a plant cutin mimetic polymer

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ABSTRACT

A mimetic polymer of plant cutin have been synthesized from 9,10,16-trihydroxyhexadecanoic (aleuritic) acid through a low temperature polycondensation reaction. Reaction conditions (solvent, catalyst, temperature, etc...) were studied and modified to optimize yield and product characteristics. The resulting polyaleurate polymer was characterized by Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR), Differential Scanning Calorimetry (DSC), X-ray Diffraction (XRD) and solid state ¹³C-Cross Polarization/Magic Angle Spinning Nuclear Magnetic Resonance (¹³C-CP/MAS NMR). Mechanical and hydrodynamic properties were also investigated. In the average, the product obtained is physically and chemically very similar to plant cutin (a hydrophobic polyester). However, a more detailed analysis of results reveals that polyaleurate framework is more rigid than natural cutin and with additional larger short-range ordered domains. Also, the synthetic polymer displays slightly different mechanical properties with respect to natural cutin. Additional hydrogen bonding within the framework of polyaleurate is considered to be responsible for such experimental observations.

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1. Introduction

The search for non toxic, fully biocompatible polymeric materials is the scope of many research projects. In this sense, polyhydroxyalkanoates and certain thermoplastic polyesters are the most investigated [1–4]. In parallel to purely chemical based and designed synthetic routes, alternative approaches such as the biomimetization of already existing natural polymers are becoming very attractive. This is the case for cutin.

The biopolymer cutin is the major component of the plant cuticle, the continuous and lipidic extracellular membrane that covers the aerial parts of leaves, fruits and non-lignified stems of plants. Chemically, the cutin is an amorphous polymeric network of polyhydroxylated C₁₆ and C₁₈ fatty acids monomers cross-linked by ester bonds. The physical, chemical and morphological properties of this natural polyester have been summarized and discussed in a comprehensive review [5]. Structural studies on plant cutin reveals an amorphous material with a surface texture that can be described as an interlaced network of “worm-like” features [6,7].

Previous progress in the biomimetization of cutin have been accomplished by its *in vitro* chemical synthesis from 9,16-dihydroxyhexadecanoic acid, the main monomer of tomato fruit cutin

[8]. The process consists in a low temperature polycondensation reaction in a hydrophobic medium [9].

In this work, a very similar monomer, aleuritic acid (9,10,16-trihydroxyhexadecanoic acid), previously used as model to study of the formation of cutin [10,11], is employed to obtain an analogous polyester. Aleuritic acid molecule contains an additional secondary hydroxyl group that is expected to improve synthesis yield and crosslinking within the polymer matrix. Chemical formulae of both 9,16-dihydroxy and 9,10,16-trihydroxyhexadecanoic acids as well as the polymers derived from their esterification are described in Fig. 1. It has been already reported ([5] and references within) that in cutin primary hydroxyls groups are preferentially involved in the ester bonds and crosslinking arises from partial participation of secondary hydroxyls. By analogy, polyaleurate is expected to retain, and even to increase, such crosslinking due to the extra secondary hydroxyl group.

2. Experimental

2.1. Chemical products and materials

Aleuritic acid and 4-dodecylbenzenesulfonic acid (DBSA) were purchased from Fluka, *p*-toluenesulfonic acid (TsOH) from Aldrich and Amberlyst[®] 15 ion-exchange resin from Sigma. Organic solvents such as toluene, chloroform, tetrahydrofuran (THF) and methanol (from Merck, analytical degree) were used without further purification.

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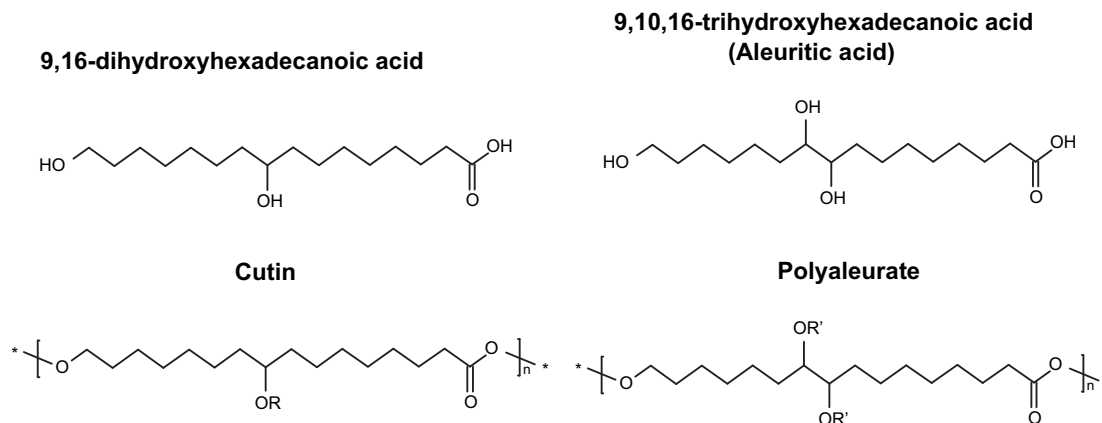


Fig. 1. Chemical structures of 9,16-dihydroxyhexadecanoic acid (the main monomer of tomato fruit cutin), aleuritic (9,10,16-trihydroxyhexadecanoic) acid, plant cutin and polyaleurate polymer. In the latter two cases R and R' represent either H or another cross-linked fragment.

Cutin samples (from leaf or fruit plant cuticles) were isolated as is described in reference [7].

2.2. Synthesis of polyesters

The polycondensation reaction between molecules of aleuritic acid was carried out similarly to the one described by Saam [9]. In a typical run, about 230 mg of aleuritic acid, 80 mg of the surfactant-type catalyst DBSA and 5 mL of toluene were introduced in a 50 mL erlenmeyer. The mixture was briefly heated to about 100 °C under continuous stirring until solids are dissolved, and then cooled to 80 °C. Minor losses of organic solvent by evaporation were corrected with the addition of small amounts of toluene. In these optimized conditions, the polyaleurate polymer began to precipitate in about 20 min forming a condensed solid layer at the bottom of the erlenmeyer. After 3 h the reaction was stopped. The solid was filtered, washed sequentially three times with toluene, chloroform and methanol and dried at room temperature for 24 h. The result was a brown plated rubber-like film that is insoluble in every solvent tried. Alternatively, in some experiments, small amounts (6–12 mg) of some powdered polymers like cutin, apple pectin or polyaleurate were added to the reaction mixture as primers to induce precipitation.

2.3. Characterization

ATR-FTIR infrared spectra of samples were obtained with an ATR accessory (MIRacle ATR, PIKE Technologies, USA) coupled to FTIR spectrometer (FT/IR-4100, JASCO, Spain). All spectra were recorded in the 4000–600 cm^{-1} range at 4 cm^{-1} resolution and 250 scans were accumulated.

^{13}C -CP/MAS NMR experiments were carried out using a Bruker DMX-400 spectrometer operating at a ^{13}C frequency of 100.62 MHz and equipped with a double resonance H/X CP-MAS 4 mm probe. The MAS rate was fixed at 5000 Hz and each experiment was recorded at ambient temperature (294 ± 1 K).

The Cross Polarization pulse sequence used a 3.5 μs 90° proton pulse, a 300 μs contact time at 62.5 kHz and a 2.5 s recycle time for an acquisition time of 17 ms during which dipolar decoupling (TPPM) was applied. Chemical shifts were calibrated with external glycine, assigning the carbonyl carbon at 176.03 ppm.

Measurements of the proton and carbon rotating-frame relaxation times $T_{1\rho}$ were achieved using a delayed-contact experiment by varying the spin-locking pulse delay according to Newman and Hemmingson [12]. The radiofrequency power level of the spin-locking pulse was fixed at 62 and 83 KHz for proton and carbon $T_{1\rho}$ measurements respectively.

XRD diffraction patterns were obtained with an X'Pert Pro (PAN-alytical) automated diffractometer using Ge(111)-monochromated $\text{CuK}\alpha$ radiation and an X'Celerator detector. The diffractograms were recorded between 5° and 45° (2θ) in 0.017° steps at 45 kV and 35 mA for 30 min. The samples were placed on an aluminium support adapting them to the goniometer in a θ - 2θ configuration.

DSC characterization of the polymer was done with a differential scanning calorimeter (DSC-50, Shimadzu Corp.). Scan rate was 5 °C per minute in the –60 to 100 °C temperature range. The low temperature end was reached with liquid nitrogen. Aluminium DSC pans contained about 4–6 mg of sample.

Water permeability of polyaleurate was determined using a methacrylate transpiration chamber as the one previously described by Schönherr and Lenzian [13], in which the solid polymer is sealing the end of a donor compartment filled with water. The transpiration chamber was kept at 25 °C inside a closed box containing silica gel. Water diffusing from the donor compartment through the polymer into the silica gel was monitored by weighting the chamber with a microbalance at regular time intervals.

Water adsorption capacity of the polymer was obtained gravimetrically. Amounts between 25 and 60 mg were introduced for 24 h in a closed chamber at 95% relative humidity. This is achieved using a vessel containing a supersaturated solution of Na_2HPO_4 at 25 °C.

Mechanical tests. The mechanical properties of the polymer were measured using an extensometer equipped with a linear displacement transducer (Mitutoyo, Kawasaki, Japan) (resolution of ± 1 μm). The equipment is the one described by López-Casado et al. [14]. Rectangular uniform pieces (3 mm \times 9 mm and about 300 μm thick) were cut directly from the obtained films and inspected microscopically to check for the absence of small cracks before mechanical testing. Every sample was maintained inside the extensometer chamber at least 30 min for temperature and humidity stabilization before any experiment. The mechanical tests were performed as a transient creep stage to determine the changes in length by maintaining samples in uniaxial tension under a constant load for 1200 s. The corresponding stress–strain curves were obtained by plotting the applied stress versus the relative total length modification. Stress–strain curves were repeated from five different samples at 23 °C and 40% RH to ensure reproducibility.

3. Results

3.1. Polyester synthesis

A considerable amount of work has been done for the determination of the reaction conditions leading to the best results in the

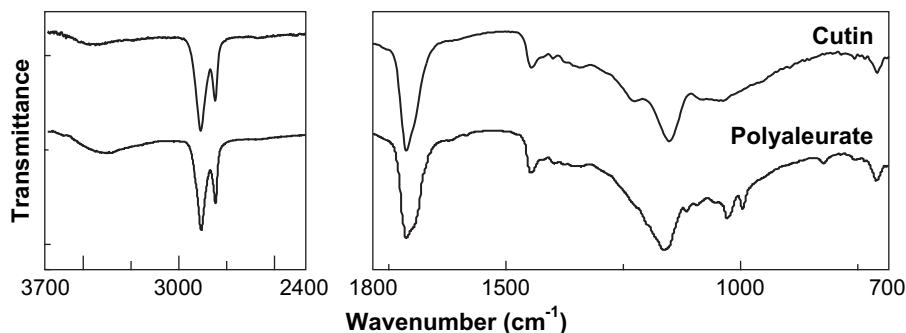


Fig. 2. ATR-FTIR spectrum of purified native cutin isolated from tomato fruit cuticles and synthetic polyaleurate. Main differences are associated to the presence of extra hydroxyl groups in polyaleurate.

polymerization of aleuritic acid. The starting point is the one described in reference [8]. Here, temperature, solvent, type of catalyst and primer and relative amount of each component have been optimized to improve reaction results. The optimization process is described in the following paragraphs and summarized in a table included as supporting information.

Regarding the catalyst used, a flexible, rubber-like brown continuous thin film is obtained using DBSA. However, with TsOH and Amberlyst® 15, small fragments of the same type of product are generated. The reaction yield, defined as mg of polyester per 100 mg of aleuritic acid, is: 67.9 for DBSA, 60.9 for TsOH and 9.9 for Amberlyst® 15.

Several reaction temperatures were tried: 50, 60, 70, 80 and 90 °C in toluene (b.p. 111 °C). At lower temperature, 50 and 60 °C, aleuritic acid did not fully dissolve in toluene and remained as a suspension, consequently, the polymerization did not take place. At 70, 80 and 90 °C the reaction rate increased with temperature. However, at 70 and 80 °C the polyester precipitated as an homogeneous thin film while at 90 °C, a chaotic growth originated a misshapen bulky product.

Three solvent have been tried and compared: toluene, chloroform and tetrahydrofuran (THF). With toluene the reaction was carried out at 80 °C, whereas with THF and chloroform the temperature had to be reduced to 55 °C because of their lower boiling point. In these later cases, reaction rate was too low to become an alternative to toluene.

Once the catalyst, temperature and solvent have been selected, the next step was to change the amount of DBSA added. Weights of 10, 40, 80 and 120 mg (per 230 mg of aleuritic acid) have been tried. With 10 mg of DBSA the reaction did not take place within the first 3 h. With the addition on increasing amounts of DBSA the reaction rate increased. The polymer presence was detected in ten minutes using 120 mg of DBSA, in 20 min for 80 mg and approximately in 35 min with 40 mg. Meanwhile, reaction yields were very similar: 59.2, 67.9 and 53.1, respectively.

The influence of primers in the polymerization of aleuritic acid was also studied. In standard reaction conditions, the addition of isolated and purified leaf *Clivia miniata* cutin produced 68.6 mg of polyester per 100 mg of aleuritic acid. When apple pectin and polymerized aleuritic acid were added, 46.5 mg and 47.1 mg were respectively obtained.

In consequence, the best reaction results are obtained using toluene as solvent, 80 °C reaction temperature, DBSA (80 mg per 230 mg aleuritic acid) as catalyst and *Clivia miniata* leaf cutin as primer.

3.2. Characterization

Fig. 2 compares the ATR-FTIR spectra of cutin isolated from tomato fruit cuticles and synthesized polyaleurate. Both spectra are quite similar. Intense bands at 1730 cm⁻¹ are fingerprints of C=O

vibration in esters (–COOR). Besides, broad bands around 3400–3450 cm⁻¹ are representative of hydrogen bonding hydroxyl groups. Main ATR-FTIR peaks are compiled in Table 1 for both substances.

The ¹³C-CP/MAS NMR spectra, Fig. 3(a), corroborate ATR-FTIR results. The signals between 24 and 34 ppm are assignable to methylene carbon (CH₂), the resonances at 64 and 72 belong to the carbons CH₂–OCOR and CH–OCOR or CH–OH respectively and, finally, the peak at 173 ppm, to the ester carbon R–COO–R [7]. A small peak at 105 ppm is assigned to aromatic carbons coming from residues of the DBSA catalyst in polyaleurate and trapped flavonoids molecules in natural cutin. Besides, chain mobility in both polymers was determined by measuring the spin-lattice relaxation in the rotating-frame (T_{1ρ}) of the methylene carbon and hydrogen atoms. Results are shown in the Table 2.

X-ray diffraction patterns of polyaleurate and natural cutin are also quite similar, Fig. 3(b). The broad band at 0.46 nm is characteristic of a mostly amorphous polymer.

The thermogram of polyaleurate, Fig. 4, shows three thermal features: a glass transition around –1.9 °C and two small endothermic events around 46.0 and 72.8 °C, that can be related with the presence of larger short-range ordered domains.

3.3. Hydrodynamic and mechanical properties

Water adsorption of polyaleurate from a water saturated atmosphere was (4.0 ± 0.3) % relative to the weight of the dry sample. The water permeability (permeance) calculated was (2.6 ± 0.4) × 10⁻⁵ m s⁻¹. Both parameters are representative of a hydrophobic material.

Fig. 5 displays typical mechanical stress-time (5a) and stress-strain (5b) curves of polyaleurate at 23 °C and 40% relative humidity. A biphasic behavior can be observed as defined by an elastic and a viscoelastic regime. From the elastic part of the stress–

Table 1

Main ATR-FTIR absorption bands observed for polyaleurate and natural cutin polymers. In both cases, spectrum is typical for an aliphatic polyester.

Assignment ^a	Wavenumber (cm ⁻¹) (Intensity ^b)	
	Cutin	Polyaleurate
ν(O–H...O)	3444 (w, br)	3403 (m, br)
ν _a (CH ₂)	2925 (s)	2925 (s)
ν _s (CH ₂)	2853 (m)	2854 (m)
ν(C=O) ester	1729 (vs)	1730 (vs)
δ(CH ₂) scissoring	1462 (w)	1463 (w)
ν _a (C–O–C)	1169 (vs)	1178 (vs)
ν _s (C–O–C)	1103 (w)	1108 (w)
δ(CH ₂) rocking	724 (w)	725 (w)

^a ν: stretch, δ: deformation, a: asymmetric, s: symmetric.

^b s: strong, m: medium, w: weak, v: very, br: broad, sh: shoulder.

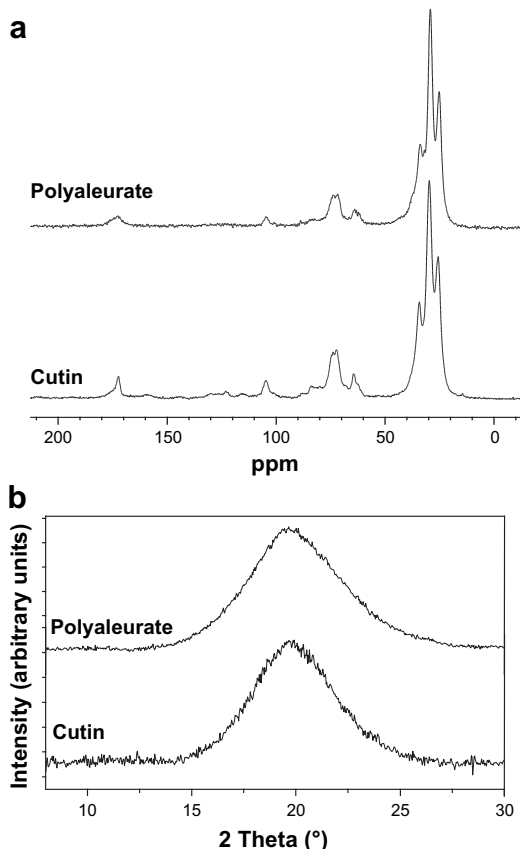


Fig. 3. (a) ^{13}C -CP/MAS NMR spectra for polyaleurate and cutin polymers. The peak at 173 ppm is indicative of ester groups. (b) XRD revealing their amorphous character. In both cases, no major differences can be appreciated by these characterization techniques.

strain curve, the elastic (E) modulus of polyaleurate polymer is calculated ($E = 17 \pm 3$ MPa).

4. Discussion

4.1. Synthesis method

The chemical reaction acting in the polycondensation of aleuritic acid is a low temperature esterification in an organic media in which the by-produced water molecule is transferred from the hydrophobic phase to the hydrophilic one. The free energy released in the transference becomes the driving force for the overall process [9]. The role of the catalyst is to trap water molecules from the reaction media and to prevent the inverse reaction of ester hydrolysis. DBSA is a surfactant organic acid and its capability to form inverse micelles, in whose polar interior water molecular are captured, is proposed to explain its participation in the reaction. This explanation accounts for our experimental result showing that the synthesis is faster with increasing amounts of DBSA. Literature

Table 2
Spin relaxation time for methylene C and H atoms in synthetic polyaleurate and natural cutin.

δ (ppm)	Polyaleurate		Cutin	
	$T_{1\rho}$ C (ms)	$T_{1\rho}$ H (ms)	$T_{1\rho}$ C (ms)	$T_{1\rho}$ H (ms)
24.8	2.44 ± 0.09	1.44 ± 0.03	3.72 ± 0.42	2.16 ± 0.09
(CH) ₂	2.45 ± 0.05	1.49 ± 0.04	4.30 ± 0.14	2.78 ± 0.24
33.5	1.46 ± 0.17	1.54 ± 0.01	2.56 ± 0.21	2.31 ± 0.22

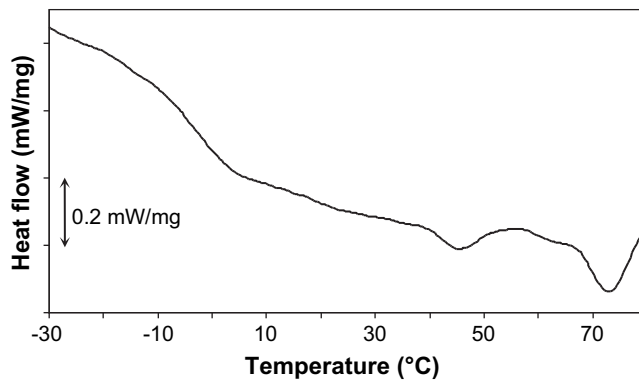


Fig. 4. DSC thermogram of polyaleurate. Glass transition is observed at -1.9 °C. Additional endothermic peaks are detected at 46.0 °C and 72.8 °C.

data [15] about the role of DBSA in esterification reactions indicates that better reaction yields are achieved with smaller amounts of this catalyst. The argument proposed is that a higher concentration of DBSA generates smaller micelles, thus increasing their external surface and the probability of water release from their core. In our case such effect is no observed, the best yield results are obtained for intermediate concentrations. We think this is due to a balance between size and number of micelles produced in our specific reaction conditions. Other acid catalysts such as TsOH and Amberlyst[®] 15 provide worse results, which are likely related to their inability to form inverse micelles.

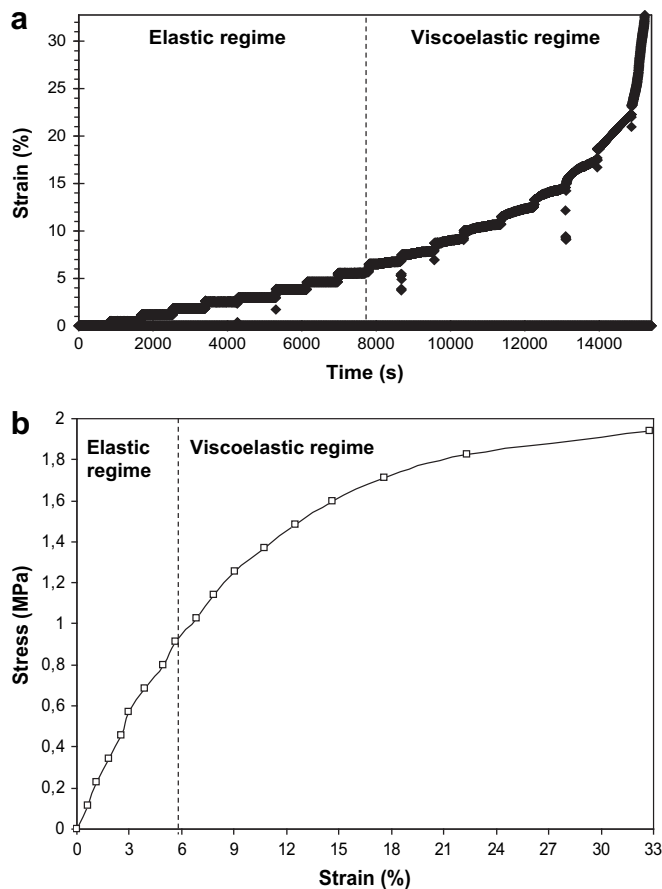


Fig. 5. (a) Characteristic strain vs time and (b) corresponding stress–strain curves of synthetic polyaleurate films. Data are obtained at 23 °C and 40% relative humidity. The presence of an elastic and a viscoelastic regime are detected.

The same explanation is invoked for the influence of other parameters like temperature and type of solvent involved in the esterification reaction. Thus, elevated temperature is expected to break the interactions between surfactant acid molecules and cause the rupture of the inverse micelles with negative consequences. For the same reason, solvents with higher polarity than toluene, i.e. chloroform and tetrahydrofuran, are inadequate despite the better solubility of aleuritic acid at low temperatures. In these solvents, the formation of the non-polar inverse micelles is disfavored and the probability of hydrolysis increases.

The use of primers (polyaleurate, isolated *Clivia miniata* cutin and commercial apple pectin) in the synthesis reduced the reaction time and, in general, the amount of product. The justification of this phenomenon isn't trivial and a more exhaustive investigation is needed to clarify this point. Our hypothesis is that a local concentration of reactants produced at the surface of the primer, triggers the esterification reaction before the same conditions are reached in the liquid medium. The lower yield is, however, associated to a modification of the chemical state of reactants. The three primers used do have free hydroxyl groups that may affect the stability of adsorbed DBSA/water inverse micelles. If water molecules are released from them, the esterification reaction will go backwards and the formation of polyaleurate will be hindered. This explanation is consistent with the lower yield obtained using apple pectin and polyaleurate (at least two free hydroxyl groups per monomer unit) compared with *Clivia miniata* leaf cutin (one free hydroxyl group per monomer unit).

4.2. Cutin-polyaleurate comparison

The synthetic route proposed in this article has been successful in creating an aleuritic acid polyester (polyaleurate) very similar to plant cutin. Chemically, there is a good correspondence as observed by ATR-FTIR and ^{13}C -CP/MAS NMR. Such similarity is a direct consequence of the analogy between the main cutin monomer, the 9,16-dihydroxyhexadecanoic acid, and the aleuritic acid molecule (9,10,16-trihydroxyhexadecanoic acid). The only chemical difference to be noticed is the higher presence of non-esterified hydroxyl groups in polyaleurate as revealed from ATR-FTIR, Fig. 2. This is obviously due to the higher $-\text{OH}/-\text{COOH}$ ratio in aleuritic acid molecule if compared with cutin monomer. After full esterification one and two unreacted hydroxyl groups per molecule are expected for cutin and polyaleurate respectively. Those available hydroxyl groups also explain the higher water absorption of polyaleurate (4% w/w) if compared with cutin (2.5% w/w). The experimental 1.6 ratio is very close to the theoretical 1.9 deduced from the chemical formula of both molecules. Still both water absorption values are quite low, indicating highly hydrophobic polymers. This is corroborated by the extreme low water permeance value obtained.

Structurally, both plant cutin and polyaleurate are mainly amorphous. This is shown by X-ray diffractograms, Fig. 3(b), where broad bands, assignable at basal distances between methylenic chains, are identical.

However, some differences between both materials have been revealed by DSC and mechanical testing. The detection of two small endothermic peaks in the thermogram of polyaleurate (Fig. 4), not observed for cutin, indicates the presence of additional larger short-range ordered domains in the synthetic polyester. Another difference is their glass transition temperature (T_g), -47.0°C for cutin and -1.9°C for polyaleurate. The higher T_g of the synthetic polyester is associated to a lower mobility of hydrocarbons chains. This is

corroborated by spin-lattice relaxation times ($T_{1\rho}$) for C and H atoms in methylene groups, Table 2. Lower spin relaxation values indicate that polyaleurate framework is more rigid than cutin. This is probably due to a more extended hydrogen bonding network within the synthetic product.

Mechanically, both materials have very similar elastic modulus, $E = (17 \pm 3)$ MPa for polyaleurate and $E = (15 \pm 2)$ MPa for cutin [14]. However there is an important mechanical difference, while polyaleurate is a biphasic material, i.e. it displays an elastic and a viscoelastic regime (Fig. 5), natural cutin is mostly a viscoelastic material [14]. The more extended elastic region of the synthetic polymer is related to its larger hydrogen bonding network. The flexibility of rupture/formation of hydrogen bonds between monomeric units is a reservoir to accommodate deformations expanding the elastic regime before stress irreversibly affects stronger linkages.

5. Conclusion

Following the synthetic route previously described by our group [8] the chemical synthesis of a plant cutin mimetic polyester is carried out using aleuritic acid as monomer. Reaction conditions such as temperature, solvent, type of catalyst and the presence of primers were studied and optimized. The characterization of this new polyaleurate by ATR-FTIR, DSC, ^{13}C -CP/MAS NMR and XRD reveals a basically amorphous and hydrophobic polyester. Despite their chemical and short-range structural similarities, the aliphatic chain network in polyaleurate is more rigid than in natural cutin. Besides, the synthetic polymer displays a more extended elastic behaviour. Both characteristics are explained by the presence additional hydrogen bonding within the synthetic polyaleurate framework.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.polymer.2009.10.018.

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