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Glycerol-based enzymatically synthesized renewable polyesters: Control of molecular weight, degree of branching and functional endgroups

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ABSTRACT

The catalytic specificity of Lipase B from *Candida antarctica* (CALB) has been exploited to achieve specific terminal groups for the synthesis of glycerol poly(ester)s. This permits the preparation of glycerol-derived macromers able to undergo further reactions/chemical modifications for customization as functional systems and building blocks for polymer synthesis. Poly(glycerol adipate) based macromers with different terminal structures were achieved from the reaction between glycerol and divinyl adipate by varying the stoichiometry (from equimolar to 1.5 and 3.0 excess either of glycerol or divinyl adipate) and amount of catalyst (1 and 3 wt%). In the course of polymerization, transesterification and hydrolysis reactions occur over vinyl functionalities that result in acid endgroups. Thus, the resulting macromers are either terminated with hydroxyl groups and/or acid groups and/or vinyl groups. Therefore, an understanding of how acid functionality appears (from vinyl groups hydrolysis) and its quantification under various conditions may provide an opportunity for the design of synthetic strategies to avoid acid production and obtain the desired structures. Endgroup analysis was carried out using ^{1}H NMR and ^{13}C NMR spectroscopies and MALDI-TOF spectrometry. Mainly linear hydroxyl and vinyl-terminated macromers were obtained with high efficiency without significant hydrolysis of the chain ends (for vinyl terminated).

1. Introduction

Glycerol (1,2,3-propanetriol; IUPAC) is the simplest of the sugar alcohols (polyols/alditols), bearing three hydroxyl groups (trifunctional monomer). Interestingly, this biobased molecule is abundantly available on the market and can be obtained by multiple pathways: from microbial fermentation of sugars; as an inexpensive co-product of the bioethanol production as well as the hydrolysis/saponification or transesterification of fats and oils for soap and biodiesel production, respectively [1,2]. Currently, the amount of glycerol derived from biodiesel preparation is substantial and represents more than 50% of the world production of this compound: e.g. in 2014, less than 0.25% of the glycerol was petroleum-based [3]. This abundance has created a need for diversification of glycerol utilization to ensure sustainable development and an environmentally-friendly face for the chemical industry. In fact, many efforts made for building a sustainable economy around the glycerol sub-product are starting to yield positive results. It is already a relevant industrial precursor for high demanded chemicals, i.e., propylene glycol.

Additionally, polyglycerols are currently reaching a stage of industrial significance being the focus of much research and finding applications in numerous fields, such as food, cosmetic and textile industry or pharmaceutical and medical applications being perhaps, the biomedical ones the most relevant [4–9]. Thus, as is evident, this surplus of glycerol is stimulating a wide range of research and development activities. Within this environment the synthesis of new glycerol containing macromers may provide the basis for new value-added applications in different fields.

For achievement of structural homogeneity in polymers, copolymers and macromers formed from glycerol and any other multifunctional sugar alcohols, strict control of synthesis is needed to control branching and avoid early gelation in these systems [10,11]. Recently, it has been demonstrated that nongelling hyperbranched polyglycerol polymers may be obtained using the Macosko-Smith statistical model for

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determining the ratio of monomers to be used during polycondensation of glycerol and adipic acid at high temperature [12,13]. Alternatively, common practice has been to selectively protect/block hydroxyl groups in order to achieve linear derivatives able to be used in further polymerization or modifications [14]. A better alternative although less exploited is to use selective enzyme catalysis such that a preference for reaction at particular hydroxyl groups can be achieved [15–17]. Thanks to the regioselectivity induced by these catalysts, linear polyesters/macromers from polyols, and diacids or their esters have been successfully obtained, avoiding gelation due to esterification at secondary hydroxyl groups, under specific conditions [18–21]. The reaction of bis (enol esters) (vinyl esters) such as divinyl sebacate and, to a less extent, divinyl adipate and various diols and triols in the presence of a lipase catalyst has been previously reported and extensively investigated [22–24]. Kline et al. first reported the synthesis of glycerol polyesters from divinyl adipate using enzymatic catalysis [19]. Uyama et al. varying temperature, enzyme origin and monomer feed ratio controlled the regioselectivity in enzymatically catalysed polymerization of divinyl sebacate and triols [20]. Kallinteri et al. successfully prepared poly (glycerol adipate) polymers with several molecular weights varying the reaction time, and subsequently functionalized the pendant hydroxyl groups with different acyl substituents [25]. Taresco et al. studied the enzymatic synthesis of poly(glycerol adipate) from glycerol and divinyl adipate at different times and temperatures observing an increasing branching degree (from 5 up to ~30%) when rising the temperature from 40 °C to 70 °C [23]. All these poly(glycerol adipate) based macromers with secondary pendant hydroxyl groups can find countless applications in the biomedical field as further modification allow to introduce various acyl groups interesting i.e. for drug delivery systems [26,27].

However, there has been a lack of attention to the possibility of producing linear macromers with endgroups of a single structure in these investigations, a valuable tool as they can be available for subsequent reactions. On one hand, structural control of polymer terminal groups is advantageous since terminal-functionalized polymers, typically macromers and telechelics are very useful for synthesis of functional polymers and, therefore, of fundamental practical importance in polymer chemistry [28–30]. On the other hand, in the case of polyols the retention of pendant hydroxyl groups is also desired as can allow for modulating the hydrophilicity and degradability of obtained polymers/macromers, for further functionalization, drug-encapsulation, for conjugating a variety of active molecules that can lead to future novel materials as well as for acting as precursors of graft copolymers [19,31–34].

In this regard, the majority of biocatalytically synthesized polyesters from activated esters and diols/polyols are mainly carried out for equimolar concentrations, and show no vinyl termination, as hydrolysis of vinyl ester endgroups occurs producing acid species. Non-stoichiometric initial monomer concentrations together with catalyst concentration are expected to be the most effective way to control the formation of polyesters exhibiting majority of desired endgroups. The catalytic specificity of *Candida antarctica* lipase B (CALB) has been utilized in the present study of the effect of catalyst amount and reagent stoichiometry over hydrolysis extension and thus over terminal groups, and to obtain glycerol-derived macromers able to undergo further reaction/chemical modification. Control of endgroup functionality will provide functional systems and building blocks for polymer synthesis [32]. Poly(glycerol adipate) based macromers with varied terminal structure have been obtained in a single-step from reaction of glycerol and divinyl adipate at different stoichiometry ratios in the presence of varying amounts of catalyst. The resulting polymers have been thoroughly characterized in terms of endgroup functionality, branching degree and molecular weight, employing different techniques including ^{1}H NMR, ^{13}C NMR, MALDI-TOF and SEC. Insights regarding the structures that may be obtained through the judicious selection of stoichiometry ratios and amount of catalyst present have been gained. Further controlled functionalization of the obtained macromers will enlarge the spectrum of glycerol-based materials for advanced applications.

2. Experimental section

2.1. Materials

Lipase B from *Candida antarctica* immobilized on microporous acrylic resin (CALB 5.000 U/g, Sigma-Aldrich); glycerol (GLY, 99%, Sigma-Aldrich); divinyl adipate (DVA, 96%, TCI Europe); acetone (99.5%, Scharlau); Diethyl ether (99.8%, Honeywell) and deuterated acetone (99.8%, Eurisotop), dimethyl formamide (DMF, Scharlau), lithium bromide (LiBr, Sigma Aldrich, >99.9%) were used as received. Anhydrous tetrahydrofuran was obtained by passing analytical grade over a solvent purification system (SPS).

3. Methods

3.1. Enzymatic Synthesis of Glycerol-co-Divinyl adipate based Macromers (PGAs)

Reactions were performed under a different set of initial reagents proportions: equimolar amounts of divinyl ester and glycerol (GLY:DVA 1.0:1.0), short divinyl ester and glycerol excess (GLY:DVA 1.1:1.0 and 1.0:1.1) and large divinyl ester and glycerol excess (GLY:DVA 1.5:1.0; 3.0:1.0; 1.0:1.5 and 1.0:3.0). Reactions were carried out following a protocol adapted from Kallinteri et al. [25] Briefly, appropriate amounts of DVA and GLY were dissolved in anhydrous THF (730 mg/mL relative to monomers weight) for about 30 min into previously dried reactor tubes (glass reactor vials of 150 mm × 24 mm ø, total volume 20 mL). A known amount of CALB (1 or 3 wt% relative to monomers weight) was added to the reaction mixture, and vials were placed in a parallel synthesizer (Carousel 12 Plus Reaction Station, RR91091, Radleys). The reaction mixtures were maintained at 40 °C under argon atmosphere while stirring at 200 rpm during fixed periods of 6 h or 24 h. After the set time, reactions were finished with the addition of cold THF, in order to dilute the products and facilitate the filtration of the enzyme. Products after filtration were concentrated and dried using a rotary evaporator, dissolved in acetone, precipitated into an excess of cold diethyl ether and recovered by filtration. Obtained macromers were dried under vacuum until constant weight was obtained and no residual solvents were detected during characterization. To assess the reproducibility of the results, reactions were repeated twice. A blank reaction was also performed for equimolar amounts of DVA and GLY without CALB, to ensure the need of the catalyst for the reactions. No conversion of the monomers was obtained. Temperature, solvent and monomer concentration were chosen based on previous reported works in order to ensure the miscibility of the monomers as well as the highest possible linearity of the obtained macromers/prepolymers [19,23,25].

3.2. Characterization

Size Exclusion Chromatography (SEC). The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity index (Đ) of the obtained polyesters were determined by SEC using a Waters Division Millipore system equipped with a Waters 2414 refractive index detector. DMF stabilized with 0.1 M LiBr was used as eluent at a 1 mL min^{-1} flow rate and a temperature of 50 °C. Styragel packed columns (HR2, HR3, and HR4, Waters Division Millipore) were used. The calibration curve was obtained using poly(methyl methacrylate) standards (Polymer Laboratories LTD).

Nuclear Magnetic Resonance (NMR). ^{1}H (128 scans, 1 s relaxation delay) and ^{13}C NMR (5120 scans, 1 s relaxation delay) spectra were recorded in deuterated acetone (≃40 mg/mL for ^{1}H NMR and ≃80 mg/mL for ^{13}C NMR) on a TM Bruker DPX 400 (400 MHz) spectrometer using tetramethylsilane (TMS) as internal reference for reported

chemical shifts.

3.2.1. Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) mass spectrometry

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) measurements were performed on a Voyager-DE PRO time-of-flight mass spectrometer (Applied Biosystems) equipped with a nitrogen laser emitting at $\lambda = 337$ nm. Spectra were acquired in positive ion mode and delayed extraction and instrumental settings were tuned to parameters that optimized signal intensity and resolution. 1 μL of matrix (2,5-dihydroxybenzoic acid (DHB)) and 1 μL of caesium chloride were mixed with 1 μL of each sample (1 mg/mL) dissolved in acetone.

4. Results and discussion

To investigate how polymerization proceeds, reactions varying either the GLY:DVA monomers' ratio or the enzyme concentration, were performed.

4.1. Enzymatic synthesis of PGAs

The research here is focused on the enzymatic synthesis and characterization of poly(glycerol adipate) (Scheme 1) with varied structure (molecular weight and degree of branching) and end functional groups. Of particular interest is to study the possibility to easily control both, structure and endgroups through catalyst amount, time, and stoichiometry.

Stoichiometry of the substrates has previously been reported to be important in determining the molecular weight of polyesters obtained by transesterification of DVA using CALB as catalyst [35]. In general, controlling macromers molecular weight and endgroup functionality is a valuable tool of particular importance as they can be available for subsequent reactions such functionalization, covalent attachment of specific molecules, directly crosslinked into networks or used as polyols for urethane manufacture [36]. However, most of the synthesized polyesters are obtained for equimolar amounts of reagents without intending to obtain specific end-capped functionalities [35]. Therefore, in the present study the GLY:DVA stoichiometry was varied in the presence of 1 and 3 wt% of CALB in order to profusely analyse the influence over molecular weight and endgroup functionality.

4.2. Short reagent excess

4.2.1. Influence of reagent ratios and catalyst concentration over molecular weight

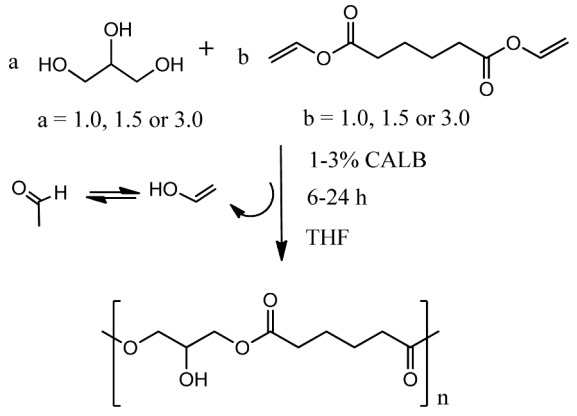
Carrying out the reactions in the presence of a short excess of reagents (1.1) has a marked effect on M_n, M_w and Đ when compared to stoichiometric conditions, as well as on the formation of triacylglycerol units in different proportions. The effects on M_n, M_w and Đ in the reaction between GLY and DVA in THF solution (60 mg/mL) are shown in Table 1.

The highest molecular weight (M_n and M_w) with CALB at 1 wt% concentration is obtained when DVA is used in excess (GLY:DVA ratio 1.0:1.1). This is consistent with the fact that DVA has a higher reactivity and, for slight excess, near to the equimolar concentrations, polymerization proceeds faster between the diester and formed oligomers at any time, compared to when glycerol is in excess. However, by increasing the amount of catalyst to 3 wt%, the highest molecular weight (M_n and M_w) is obtained for equimolar concentrations of triol and diester. It seems that the increment of catalytic activity and the existing excess of DVA, increase the rate and conversion of transesterification [37,38], leading to a consumption of DVA to a greater extent in branching reactions (reacting with secondary hydroxyl groups of GLY), instead of main chain growth. As expected, and in agreement with previous studies, the molecular weight of the polyesters increases with concentration of catalyst does [39]. Interestingly, a slight increase in the amount of catalyst, from 1 to 3 wt%, has a noticeably impact into the molecular weight. A 1.5-fold enhancement is observed in polyesters synthesized from a divinyl adipate, while equimolar and glycerol ones triplicates their molecular weight.

4.2.2. Influence of reagent ratios and catalyst concentration over endgroup functionality, branching degree and regioselectivity of enzyme

4.2.2.1. ^{1}H NMR study. Some previous investigations demonstrate the potential of ^{1}H NMR technique in the analysis of the endgroups of low molecular weight polymers [40,41]. Therefore, as a preliminary approximation, the endgroup analysis was performed in this study by ^{1}H NMR. Fig. 1 shows the assignments for terminal glycerol protons and glycerol units contained in linear and dendritic polymer chains. Fig. 2a and b shows representative ^{1}H NMR spectra of resulting polymers from equimolar concentrations of reagents, after 24 h of reaction in THF at 40 °C in the presence of 1 and 3 wt% of CALB, respectively. ^{1}H NMR spectra of polymers obtained with GLY and DVA excess in the presence of 1 and 3 wt% of CALB are shown in Supporting Information in Figs. S1 and S2, respectively. Fig. 2a shows clear visible vinyl proton signals at 7.29, 4.87 and 4.59 (p, o, o′) while, these signals become less pronounced when 3 wt% of catalyst is employed (Fig. 2b). This confirms higher presence of vinyl endgroups when 1 wt% of catalyst is employed in agreement with the lower M_n and M_w achieved in this polymer. Glycerol terminal units give multiplets in the region of 3.5–3.9 ppm also with higher intensity when lower amount of catalyst is employed (1 wt% of CALB). CH_2 adipic protons (a, b) appear in the spectral range between 1.6 and 2.5 ppm, while all protons related to glyceride repeating units are found between 3.5 and 4.5 ppm. Peaks at 5.1 and 5.3 ppm are related to 1,2-substituted (e′), and 1,2,3-substituted glycerides (dendritic, e″), respectively, being the latest produced due to the lack of regioselectivity of the enzyme during the reaction.

Therefore, the amount of 1,2,3-substitued (branching degree) and 1,2-substituted units can be calculated from ^{1}H NMR following the **Eqs.**



Scheme 1. Synthesis of PGA macromers using different ratios of reagents.

Table 1
Effect of enzyme concentration and substrate stoichiometry (40 °C, 24 h, THF).

GLY:DVA (Ratio)	CALB (wt%)	[a]M_w (g/mol)	[a]M_n (g/mol)	[a]Đ M_w/M_n	Yield (%)
1.0:1.0	1	9100	6100	1.5	65
1.0:1.0	3	26,600	16,500	1.6	65
1.1:1.0	1	3400	2600	1.3	50
1.1:1.0	3	9700	8000	1.2	65
1.0:1.1	1	9600	6700	1.4	65
1.0:1.1	3	13,600	9700	1.4	52
1.0:1.0	[(b)]-	–	–	–	0

[a] Determined by SEC Control experiment.

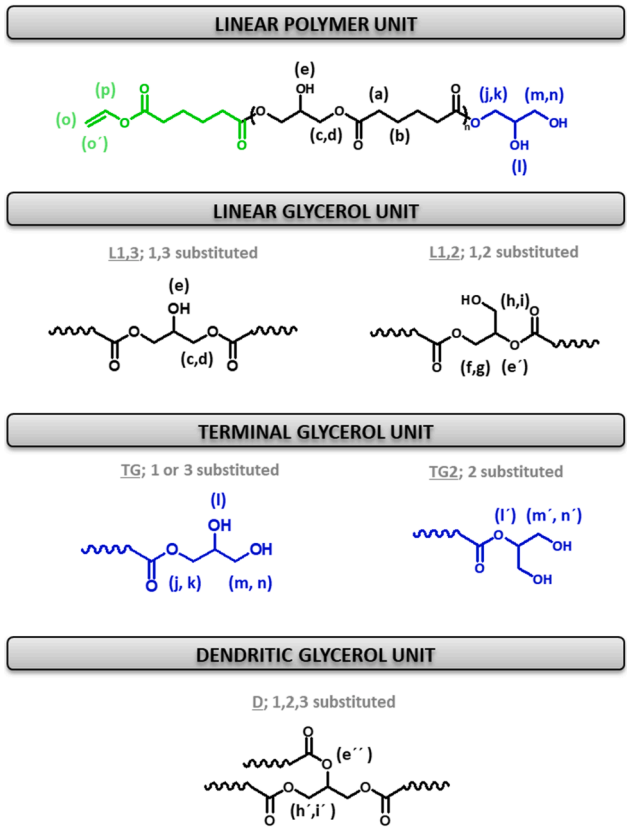


Fig. 1. ^{1}H NMR proton assignments for linear, terminal and dendritic glycerol units contained in the polymers.

(1) and (2).

$$1,2,3-substitutedunits = \frac{Ie^{''}/ne''}{Ia/na}\hat{A}\cdot 100 \quad (1)$$

$$1,2-substitutedunits = \frac{Ie^{'}/ne'}{Ia/na}\hat{A}\cdot 100 \quad (2)$$

where *I* is the intensity and *n* is the number of protons.

Table 2 shows the relative abundance of glycerol and vinyl endgroups depending on the amount of catalyst and reagent ratios (see Supporting Information Table S1 and Eqs. (S1)–(S16)). According to the data collected in Table 2, the relative abundance of GLY and DVA endgroups of each polymer varies significantly according to the molar ratio used. From the results can be seen that the amount of endgroups is related to the reagent in excess (either GLY or DVA); thus, a higher proportion of GLY endgroups are formed when GLY is used in excess. Similarly, a higher proportion of vinyl endgroups seems to be achieved when DVA is the reagent in excess. This fact is more pronounced when the enzyme concentration increases from 1 to 3 wt% for both, GLY and DVA excess polymers. For equimolar concentrations of initial monomers and low concentration of catalyst (1 wt%), the relative amount of vinyl and glycerol endgroups is nearly balanced (60:40); however, an increase of enzyme to 3 wt% clearly favours the presence of glycerol endgroups.

In order to determine whether a change in stoichiometry or enzyme concentration will generate branched structures, the degree of branching (DB) of the polymers was also assessed by ^{1}H NMR and their values are also collected in Table 2 (see also Table S1 and Eq. (S17)). Degree of branching is directly related with the formation of dendritic structures, thus, is a measure of the dendritic character of the polymeric structures. Interestingly, the synthesis conditions employed retains the degree of branching to a minimum, in agreement with the high regioselectivity values obtained for all the reactions what indicates that mainly linear structures are formed. Regioselectivity of lipases towards primary alcohols in polyols has been reported before to occur within a particular range of time and temperature. Importantly, for the conditions chosen in this study, this regioselectivity seems to not be affected by slight deviations from stoichiometry or the amount of catalyst chosen. Regioselectivity values of CALB towards acylation of primary OH groups of glycerol at different catalyst amounts are estimated to be in the range of 96–98% for equimolar divinyl adipate and glycerol, which is in agreement with previous observations made at similar conditions [19,23]. In general, increasing enzyme concentration is expected to lead more branched structures due to a loss of regioselectivity. Therefore, taking into account the significant enhancement observed in the molecular weight, it is remarkable that the degree of branching when increasing the amount of catalyst is retained at much lower values than expected as the conditions employed allow retaining the regioselectivity of the enzyme.

4.2.2.2. ^{13}C NMR study. In order to corroborate the ^{1}H NMR results, ^{13}C NMR were used to further analyse the glycerol-based polymers structure and the regioselectivity of the enzyme in each reaction. To this purpose, the presence of endgroups as well as of the different structural glycerol units were evaluated and discussed in comparison to ^{1}H NMR obtained results. Note that in contrast to what happen in ^{1}H NMR the signals from different glycerol structural units are not overlapped.

This fact together with the high number of scans used, in order to ensure the appearance of all the signals with sufficient intensity, allow for a more precise structural study of the different polymers obtained. Fig. 3 shows a representative ^{13}C NMR spectra of equimolar polymers obtained with 1 wt% (Fig. 3a) and 3 wt% (Fig. 3b) of CALB, at previously specified reaction conditions. ^{13}C NMR spectra of polymers obtained with GLY and DVA excess in the presence of 1 and 3 wt% of CALB are shown in Supporting Information in Figs. S3 and S4, respectively. Signals at around 172 ppm, 142 ppm and 97 ppm are assigned to the carbonyl carbons (F) and to the vinyl endgroups (O and P), respectively. Signals corresponding to the CH_2 adipic carbons (A, B) appear in the low spectral range between 35 and 25 ppm, while signals corresponding to glycerol are in the range of 80–50 ppm. Glycerol methine carbons resonating at different ppm indicate different substituted glycerol units: signals at 68.1 ppm (E) and 73.0 ppm (É), are indicative of linear glycerol 1,3 and 1,2 substituted (L1,3 and L1,2), respectively; signals at 70.8 ppm (L) and 76.3 (L′) correspond to terminal glycerol units monosubstituted in one of the primary (TG) or in the secondary hydroxyl (TG2), respectively; signal at 70.0 ppm (E″) corresponds to 1,2,3 substituted denditric units (D) in which all the hydroxyls are reacted. Interestingly, carbon spectra corroborate the presence of TG2 with the appearance of the Ĺ signal, which related proton is difficult to be observed by ^{1}H NMR (expected resonance at ~4.9 ppm), probably due to the small amount of this glycerol units together with the near resonance of vinyl proton signals at 4.9 ppm. Methylene carbons in terminal glycerol units appear at 66.2 ppm (J, K) and 64.0 ppm (M, N) for TG and, at 61.6 ppm (M′, N′) for TG2. Methylene carbons for L1,3 are visible at 61.3 ppm (H, I) and 63.2 ppm (F, G), and for L1,2 at 65.8 ppm (C, D), while for D glycerol units appear at 62.8 ppm (H′, I′). These assignments for different glycerol units are in concordance with those previously reported [13,42–44].

Spectrum in Fig. S4(b) corresponding to the polymer obtained with DVA excess and 3 wt% of catalyst, does not present the signals corresponding to terminal glycerol units acylated at one of the secondary hydroxyl groups (TG2: signals L′, and M′, N′). All ^{13}C NMR spectra of the obtained polymers show hardly visible signals corresponding to glyceride units that do not correspond to esterification of only primary hydroxyl groups, which confirms the high regioselectivity of the enzyme under the conditions employed (Table 2). Furthermore, it can also be corroborated that the polymers have low degrees of branching, since in their ^{13}C NMR spectra the E″ signal is almost negligible. On the other

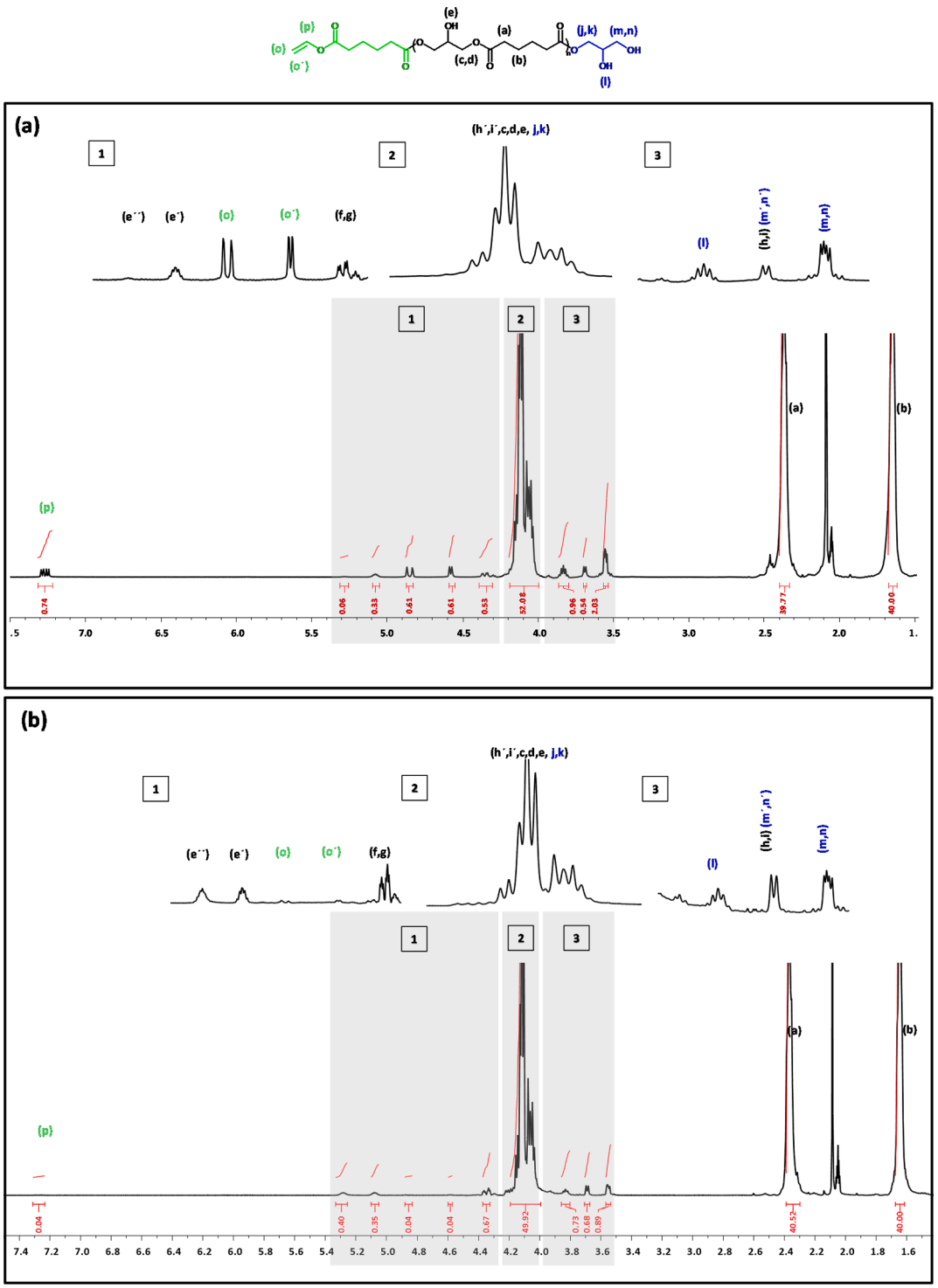


Fig. 2. ^{1}H NMR spectra of equimolar GLY:DVA syntheses at 40 °C in THF with (a) 1 wt% CALB and (b) 3 wt% CALB.

hand, around 174 ppm, very low signals appear corresponding to a carbonyl carbon of acid, which means that some percentage of the vinyl groups undergoes hydrolysis and that might be the reason why vinyl endgroups are not totally retained using the DVA excess.

Structure characterization and calculations of lipase catalysed glycerol-based polyesters has been mainly done by means of ^{13}C NMR or ^{1}H NMR spectra. However, in this particular case, both, ^{1}H NMR and ^{13}C NMR complement each other and represent powerful tools to reveal all the details in order to clarify the structure of the synthesized glycerol-based polymers. ^{1}H NMR allowed for structural calculations while ^{13}C NMR facilitates verify the presence of TG and TG2 units as well as the hydrolysis of double bonds from divinyl adipate.

4.2.3. Influence of reaction time over molecular weight and polymer structure

Previous studies have reported that the reaction time has a strong effect over the molecular weight, as polymer growth in similar lipase catalysed systems occurs according to the mechanism of step polycondensation. However, studies to date remain limited about the effect of reaction time over molecular weight and glycerol structural units in

Table 2
Effect of enzyme concentration and substrate stoichiometry (40 °C, 24 h, THF) in relative abundance of different glycerol structural units, endgroup functionality and regioselectivity of the enzyme as calculated by ^{1}H NMR.

GLY:DVA (Ratio)	CALB (wt%)	Glycerol Structural Unit relative abundance (%)					Endgroup relative abundance (%)		Regioselectivity (%)	DB (%)
		TG (1-sub.)	TG2 (2-sub.)	L1,3 (1,3-sub.)	L1,2 (1,2-disub.)	D (-sub.)	GLY	DVA		
1.0:1.0	1	8.9	–	87.6	2.9	0.5	57.8	42.2	98.2	1
1.0:1.0	3	4.2	–	88.7	3.3	3.8	91.8	8.2	96.5	8
1.1:1.0	1	33.3	0.1	64.8	1.5	0.3	83.7	16.3	98.9	1
1.1:1.0	3	22.2	0.7	69.5	5.5	2.1	96.1	3.9	95.3	5
1.0:1.1	1	3.2	1.0	89.7	5.0	1.1	25.9	74.1	96.4	2
1.0:1.1	3	1.6	–	94.1	2.0	2.4	19.2	80.8	97.9	5

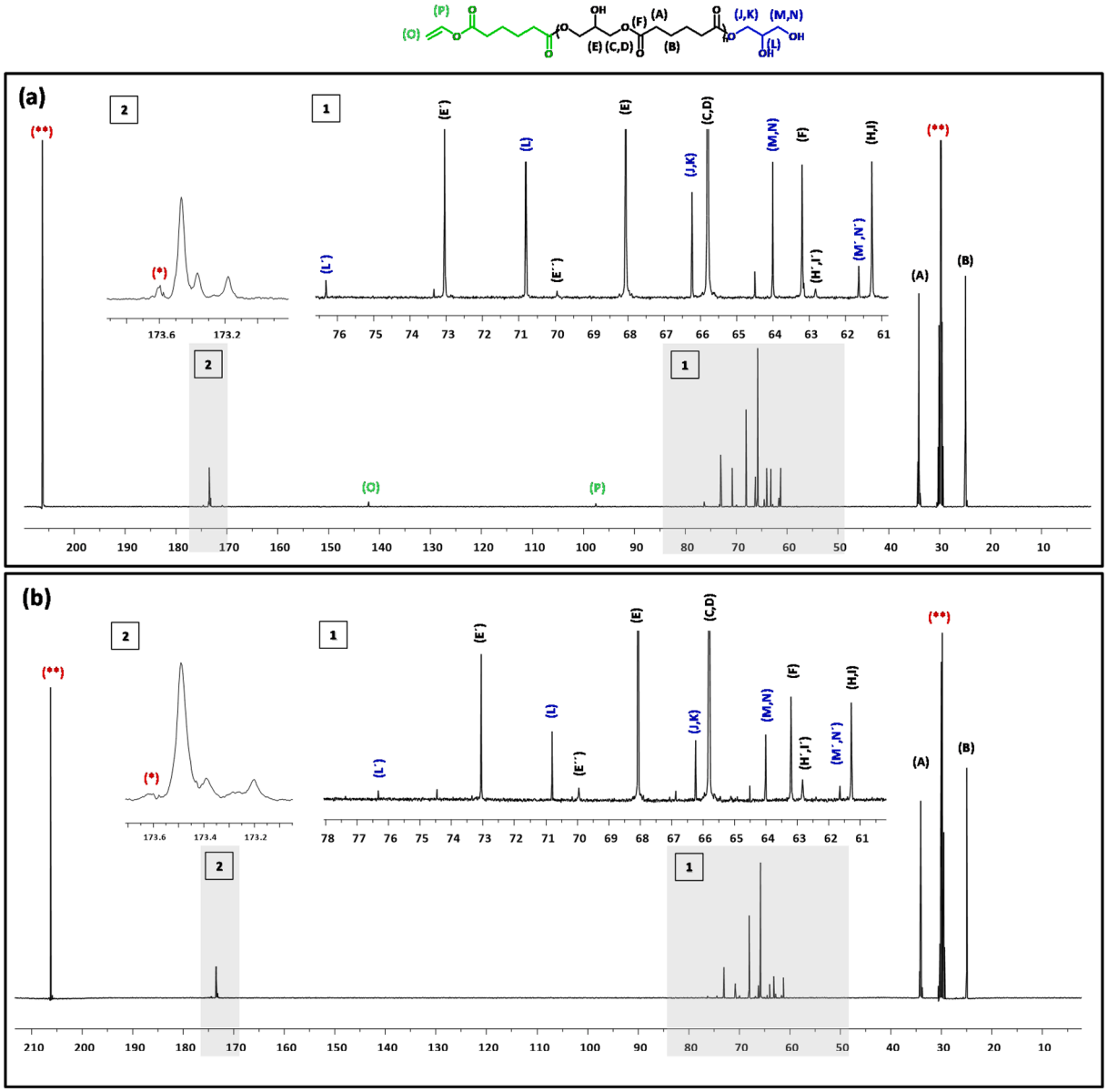


Fig. 3. ^{13}C NMR spectra of equimolar GLY:DVA syntheses at 40 °C in THF with (a) 1 wt% CALB and (b) 3 wt% CALB. (*) Adipic Acid Termination, (**) Deuterated Solvent.

glycerol based polymers. Therefore, stoichiometric and short GLY:DVA excess reactions were repeated and stopped after 6 h in order to compare the ^{1}H NMR and SEC results with those obtained after 24 h. Reactions were carried out using 3 wt% CALB, as reactions with 1 wt% CALB for such short time did not resulted in significant molecular weights [19,43,45]. The effects on M_n, M_w and Đ are shown in Table 3.

In view of the data, reaction time has a large influence over M_n and M_w. By increasing reaction time from 6 h to 24 h, a significant increase in molecular weight occurs for stoichiometric monomer concentrations. Deviations from stoichiometry and longer reaction times results in

Table 3
Effect of reaction time in molecular weight (40 °C, 6 h and 24 h, THF).

GLY: DVA (Ratio)	CALB (wt %)	Time (h)	[a]M_w (g/mol)	[a]M_n (g/mol)	[a]Đ M_w/M_n	Yield (%)
1.0:1.0	3	6	10,600	7600	1.4	64
1.0:1.0	3	24	26,600	16,500	1.6	65
1.1:1.0	3	6	10,000	7200	1.4	62
1.1:1.0	3	24	9700	8000	1.2	65
1.0:1.1	3	6	10,600	7600	1.4	66
1.0:1.1	3	24	13,600	9700	1.4	52
1.0:1.0	[(b)]-		–	–	–	0

[a] Determined by SEC Control experiment.

higher molecular weights when DVA is the monomer in excess. This is indicative that molecular weight at 6 h seems to be more sensitive to the non-stoichiometry of starting monomers, than to the reaction time as short reaction times resulted in similar molecular weights for all the systems. Table 4 shows the effect of reaction time and stoichiometry over glycerol structural units and endgroups. The regioselectivity value of lipase towards acylation of primary OH groups for equimolar amounts of divinyl adipate and glycerol at 40 °C for 24 h in THF is determined to be aprox. 97% that agrees well with previous observations made by Kline et al. [19] and Kallinteri et al. [25] that estimated the selectivity of CALB towards primary hydroxyl groups of glycerol to fall into the range of 90–95% when glycerol and divinyl reacted at almost analogous conditions to the ones employed in this work. Therefore, shallow deviations from perfect regioselectivity of the enzyme at similar temperatures and times have been reported before and the results obtained in this work perfectly match them.

Naolou et al. [46] reported perfect regioselectivity of lipase towards primary OH groups when reacting equimolar amounts of divinyl adipate and glycerol at temperatures below 60 °C for 8 h in THF, while Uyama et al. [20] obtained similar results at 45 °C in bulk. The slight differences in the regioselectivity obtained in our study at 6 h with the Uyama et al. and Naolou et al. works are probably due to the differences with the enzyme immobilization and amount, respectively.

According to the present observations the branching phenomenon has occur already at 6 h as the regioselectivity values did not increase markedly after 24 h of reaction in agreement with previous observations made by Jacob et al. [47] Therefore, extending the reaction time up to 24 h mainly affects polymer length. Interestingly, the fact that polymers obtained after 6 h possess higher amount of 2-substituted endgroups than 1-substituted seems to be directly related with the slight increase on dendritic units and branching degree occurring at longer reaction times (24 h). Thus, the free primary —OH in the 2-substituted end glycerol units continues reacting causing the formation of new dendritic units.

In relation to endgroup functionality after 6 h of reaction, the obtained data explains many observations done through the molecular weight evolution analysis. After 6 h of polymerization and equimolar amounts of reactants, although the presence of GLY endgroups predominates over DVA, the difference is not as pronounced as after 24 h. This situation allows the continuation of chain growth and, therefore, the molecular weight differences between both reaction times are the most perceptible. A glycerol excess, produces an early loss of DVA endgroups (they were almost negligible in the ^{1}H NMR spectra) after 6 h of reaction, resulting in no significant change in molecular weight for longer reaction time. Glycerol seems to cause a fairly rapid consumption of vinyl groups in chain growth, causing the disappearance of vinyl groups to that polymerization continues. After 6 h of reaction, all GLY: DVA ratios studied resulted in similar molecular weights; however, contrary to what occurs when glycerol is in excess, the higher amount of DVA allows the polymer to continue growing after 6 h. This fact is consistent with previous observations made in DVA-diol systems that state the higher reactivity of DVA with growing oligomers [48]. Thus, polymerization seems to proceed longer when DVA is in excess in comparison to what occurs when GLY is in excess. Summarizing, the increase in reaction time over 24 h allows a greater extent of polymerization for 1:1 reagent ratios, being reduced for stoichiometries other than unity. Anyway, in all the cases the polymerization proceeds without notably affecting the branching degree or the regioselectivity of the catalyst. Additionally, non-stoichiometric concentrations of starting monomers, either at 6 h or 24 h of reaction time, result in the formation of polymers exhibiting majority of endgroups from the reagent in excess.

4.2.4. *MALDI-TOF study for endgroup functionality*

In order to elucidate more details on chemical structure of enzymatically catalysed polyesters, MALDI-TOF analysis was performed. Analyses were focused in assessing quantitative chain-end functionalization of the obtained polymers at several DVA:GLY ratios as well as catalyst amount. For all the polyesters, spectra are characterized by a number of signals separated by intervals of m/z 202. These intervals correspond to the molar mass of PGA repeating unit, what confirms the marked linearity of obtained PGA under all the synthesis conditions tested, in agreement with ^{1}H NMR results. Additionally, several series of peaks are revealed indicating that the polyester PGA chains are terminated in various endgroups. The growing poly(glycerol adipate) chains can respond ending in the following functional groups: n_{gv} stands for HO–(PGA)–Vinyl ($202n + 44$), n_{vv} stands for Vinyl-(PGA)-Vinyl ($202n + 198$), and n_{gg} stands for HO–(PGA)–OH ($202n + 92$) structures. Considering that some hydrolysation of vinyl groups can have place during the reaction, the following endgroups have to also be considered: n_{ga} stands for HO–(PGA)–Acid ($202n + 18$), n_{aa} stands for Acid-(PGA)-Acid ($202n + 146$), and n_{va} stands for Vinyl-(PGA)-Acid structures ($202n + 172$). Wherein, n is the number of peaks per polymer chain.

Fig. 4 shows a representative MALDI-TOF spectrum of a poly(glycerol adipate) produced from the reaction of glycerol and excess DVA (Gly:DVA 1:1.1) in the presence of 1 wt% CALB.

The effect of reagents stoichiometric ratio together with the amount of enzymatic catalyst are known to be the most important variables affecting the polyester functionality and thus, endgroup composition. Fig. 5 shows the composition of polyesters obtained at GLY:DVA equimolar ratio (1.0:1.0), with GLY excess (1.1:1.0) and with DVA excess (1.0:1.1) under the presence of 1 and 3 wt% of enzymatic catalyst. It can be surmised that when divinyl adipate is the monomer in excess, a higher proportion of Vinyl-(PGA)-Vinyl (% n_{vv}) type chains will be

Table 4
Effect of reaction time and substrate stoichiometry (40 °C, THF) in relative abundance of different glycerol structural units, endgroup functionality and regioselectivity of the enzyme calculated by ^{1}H NMR.

GLY:DVA (Ratio)	Time (h)	Glycerol Structural Unit relative abundance (%)					Endgroup relative abundance (%)		Regioselectivity (%)	DB (%)
		TG (1-sub.)	TG2 (2-sub.)	L1,3 (1,3-sub.)	L1,2 (1,2-disub.)	D (-sub.)	GLY	DVA		
1.0:1.0	6	0.5	2.9	90.9	2.8	2.8	67.4	32.6	96.9	7
1.0:1.0	24	4.2	–	88.7	3.3	3.8	91.8	8.2	96.5	8
1.1:1.0	6	4.3	9.4	82.8	2.7	0.9	~100	–	96.0	3
1.1:1.0	24	22.2	0.7	69.5	5.5	2.1	96.1	3.9	95.3	5
1.0:1.1	6	–	0.9	96.4	1.4	1.7	7.8	92.2	98.7	3
1.0:1.1	24	1.6	–	94.1	2.0	2.4	19.2	80.8	97.9	5

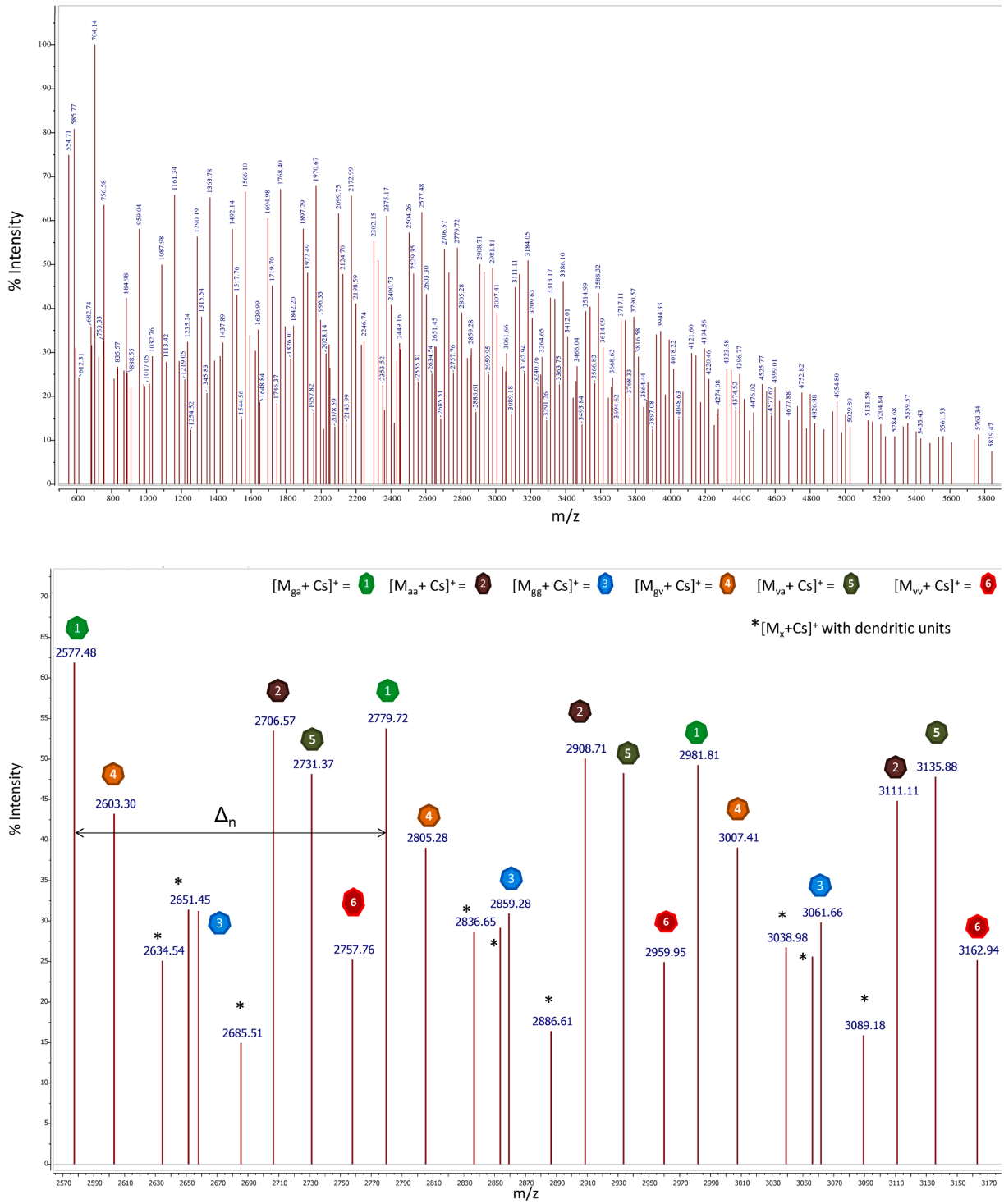


Fig. 4. (a) Full MALDI-TOF spectrum for poly(glycerol adipate) synthesized from GLY:DVA ratio 1:1.1 and 1 wt% CALB (b) Detailed region from 2500 to 3200 *m/z*.

formed; similarly, when glycerol is used in excess, a higher proportion of HO–(PGA)–OH (% n_{gg}) chains are expected. However, whereas deviation of stoichiometry in favour of excess glycerol seems an adequate approach for endgroup functionality control, the results show that for the DVA excess case, residual hydrolysis leads to the production of high percentage of acidic containing species in detrimental of ester endgroup containing ones, in contrast to the obtained ^{1}HNMR results (see Tables 2 and 5) that not clearly show the presence of such species.

By analysing Fig. 5 and Table 5, the effect of enzyme concentration over polyesters endgroup composition can be evaluated. Increasing the enzyme concentration from 1 to 3 wt% produces the disappearance of vinyl containing species in the final products. When DVA is the reagent in excess, an evident increased proportion of Acid-(PGA)-Acid (% n_{aa}) occurs; however, in the case of glycerol excess this phenomenon is not so evident. As mentioned before, because of the increase of enzymatic catalyst concentration, the initial transesterification and hydrolysis rates will increase, thus a consumption of ester containing monomers can also have place by direct hydrolysis, hindering them to take part of a growing chain. This is in agreement with the lower molecular weight observed for polyesters deviated from stoichiometry towards glycerol excess. In

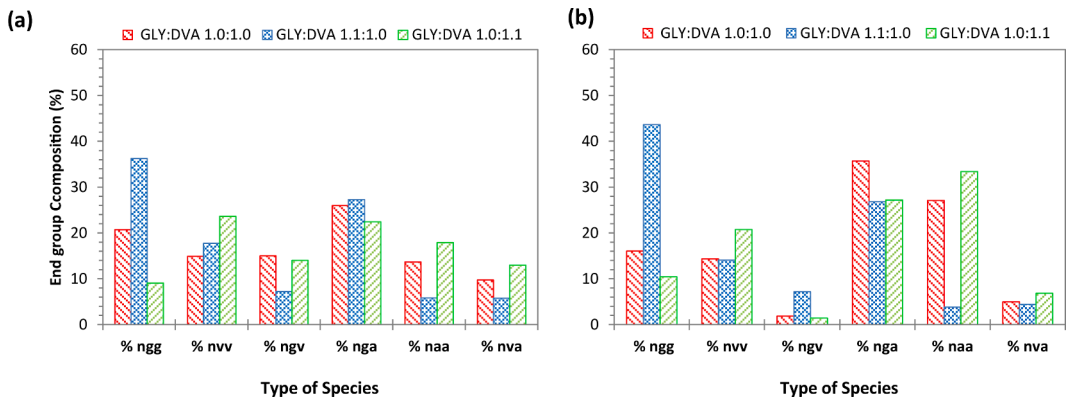


Fig. 5. Effect of substrate stoichiometry on composition of synthesized polyesters with (a) 1 wt% CALB and (b) 3 wt% CALB.

Table 5
Effect of enzyme concentration and substrate stoichiometry (40 °C, 24 h, THF) in endgroup composition relative abundance as calculated from MALDI-TOF and its comparison with ^{1}H NMR results.

GLY:DVA (Ratio)	CALB (wt%)	Endgroup relative abundance (%) MALDI TOF			Endgroup relative abundance (%) ^{1}H NMR	
		GLY	DVA	AC	GLY	DVA
1.0:1.0	1	48.0	24.8	27.2	57.8	42.2
1.0:1.0	3	41.1	17.4	41.5	91.8	8.2
1.1:1.0	1	59.2	23.0	17.8	83.7	16.3
1.1:1.0	3	66.3	18.7	15.0	96.1	3.9
1.0:1.1	1	33.4	34.8	31.8	25.9	74.1
1.0:1.1	3	29.5	24.6	45.9	19.2	80.8

this sense the higher hydrolysis rate due to the higher presence of enzyme, seems to be the most important mechanism affecting the proportion of ester containing species and, therefore, favouring the formation of HO–(PGA)–OH (% n_{gg}) species.

4.3. High reagent excess

Reactions were repeated increasing the DVA and GLY excess to 1.5 and 3.0 (GLY:DVA ratio 1.5:1.0, 3.0:1.0, 1.0:1.5 and 1.0:3.0) in THF at 40 °C under the presence of either 1 wt% or 3 wt% of CALB for 24 h. The obtained polymers were analysed by SEC, ^{1}H NMR and MALDI-TOF. Results of molecular weight, endgroup relative abundance and regioselectivity of the enzyme are summarized in Table 6.

The highest M_n is obtained for equimolar contents of reactive functionalities and 1 wt% of CALB. In connection with the results obtained for low excess reagents (1.1), higher molecular weights are obtained when DVA starting concentration is in 1.5 excess, more markedly when CALB is at 3 wt%. For equimolar and 1.5 excess obtained polymers, around 3 to 4-fold increase of M_n was obtained with increasing catalyst concentration. However, higher deviations from equimolarity (3.0 excess) resulted in a significant decrease of molecular weight and increasing the amount of CALB to 3 wt% does not produced significant mass gain. Interestingly, high deviations from stoichiometry do not increase the branching degree. Additionally, the majority of endgroups from the reagent in excess are obtained for 1.5 ratio.

Fig. 6 shows the effect of the substrate stoichiometry over endgroup functionality. When glycerol is the monomer in excess, the highest amount of OH–(PGA)–HO (%n_{gg}) end capped species (~67%) are obtained when the ratio GLY:DVA and enzyme concentration are 1.5:1.0 and 1 wt%, respectively. Increasing either glycerol or catalyst concentration results in higher acid formation and vinyl end capped species, respectively. Interestingly, an increase in glycerol content for 1 wt% of enzyme concentration results in a significant increase in OH–(PGA)-Acid (%n_{ga}) while for 3 wt% of enzyme concentration such an increase in glycerol produces more OH–(PGA)-Vinyl (%n_{gv}) end capped species.

Similarly, the highest amount of Vinyl-(PGA)-Vinyl (%n_{vv}) species in the final products (~34%) is obtained for GLY:DVA 1.0:1.5 ratio and either increasing of the amount of vinyl monomer in the reaction or the enzyme concentration do not have a significant effect or even decreases significantly the amount of Vinyl-(PGA)-Vinyl species.

5. Conclusions

Poly(glycerol adipate) macromers with varied terminal structure and molecular weights have been achieved by enzymatic catalysis in the presence of CALB at different GLY:DVA ratios and amount of catalyst. Hydrolysis of vinyl groups results in acid terminated species altering the expected structure of the obtained macromers and limiting their possibilities of been used for further functionalization. Therefore, a depth

Table 6
Effect of Enzyme Concentration and substrate Stoichiometry (40 °C, 24 h, THF) in endgroup composition relative abundance as calculated from MALDI-TOF, in regioselectivity of the enzyme as calculated by ^{1}H NMR and in molecular weight as obtained by SEC.

GLY:DVA (Ratio)	CALB (wt%)	SEC			Endgroup relative abundance (%) MALDI-TOF			^{1}H NMR	
		M_w (g/mol)	M_n (g/mol)	Đ M_w/M_n	GLY	DVA	AC	Regioselectivity (%)	Branching degree (%)
1.0:1.0	1	9100	6100	1.5	48.0	24.8	27.2	98.2	1
1.0:1.0	3	26,600	16,500	1.6	41.1	17.4	41.5	96.5	8
1.5:1.0	1	3600	3600	1.0	79.7	10.3	10.0	98.0	1
1.5:1.0	3	12,600	7700	1.6	79.2	17.2	3.6	96.5	1
1.0:1.5	1	3700	3300	1.1	30.4	44.2	25.3	98.6	1
1.0:1.5	3	15,300	8600	1.8	26.6	55.8	17.5	99.6	2
3.0:1.0	1	2000	1700	1.2	61.5	16.4	22.1	87.0	2
3.0:1.0	3	2300	1700	1.3	69.2	26.2	4.6	79.6	1
1.0:3.0	1	1700	1500	1.1	7.1	48.7	44.1	95.8	10
1.0:3.0	3	1700	1400	1.2	43.9	35.2	20.9	98.8	6

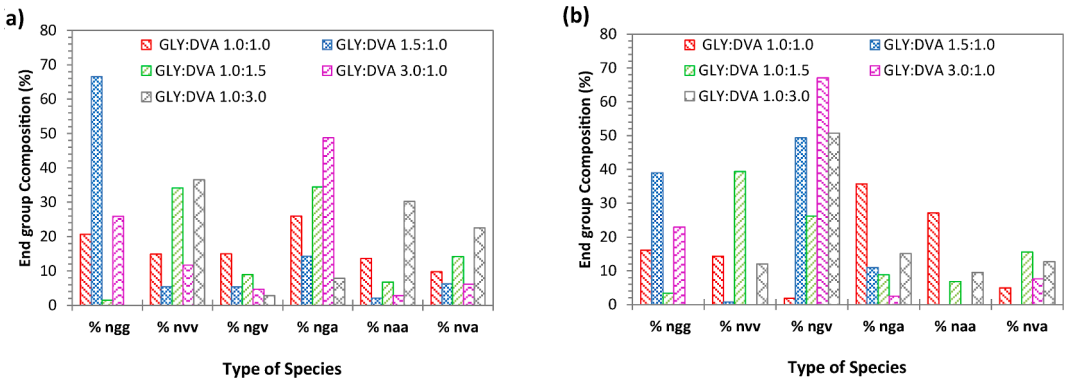


Fig. 6. Effect of substrate stoichiometry (1.5 and 3.0 excess reagents) on composition of synthesized polyesters with (a) 1 wt% CALB and (b) 3 wt% CALB.

analysis of the nature of the endgroups has been carried out. Typically, endgroup analysis for low molecular weight polymers is often performed by ^{1}H NMR, and although the knowledge of the amount of acid termination of polyesters due to hydrolysis of vinyl endgroups can be of significant value, as demonstrated, it is difficult to detect at low levels as well as due to overlapping. Thus, ^{13}C NMR analysis has been employed to confirm qualitatively the presence of acid endgroups, a result in view of ^{1}H NMR particularly surprising also due to previous works with divinyl adipate and glycerol not indicating the early hydrolysis of vinyl groups into acid form. In this line, MALDI-TOF has also been employed and provided a versatile method for endgroup analysis and acid quantification. Results have demonstrated the influence of enzyme concentration, and initial ratio of reactants over the end-functionality of the obtained macromers. Interestingly, the proportion of acid-containing chains decreases with increasing either GLY or DVA substrates amount to 1.5 excess with no significant differences with the amount of CALB. Moreover, the proportion of end hydroxyl containing species are the highest, near ~76%, for GLY:DVA 1.5 ratio, while the same excess of DVA produces ~46% of vinyl endgroups, due to hydrolysis of ester groups. Additionally, deviations from stoichiometry have a marked effect over M_n and M_w values, more noticeable for short reaction time and 3–1 wt% catalyst concentration. Besides, high regioselectivity of lipase towards primary alcohol is retained in all the conditions tested. These results suggest that by combining these strategies, that is, varying the concentration of catalyst, reaction time and stoichiometric ratios of polyol to diester open the possibility to further control the structure and endgroup presence, enlarging the variety of glycerol based macromers for further functionalization. Additionally, the understanding on how the acid group appears by the profusely characterization done, gives us the opportunity to design future synthetic strategies that avoid its production.

CRediT authorship contribution statement

Águeda Sonseca Olalla: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision, Investigation. **Víctor Hevilla Talavera:** Conceptualization, Methodology, Investigation, Validation, Investigation, Writing – original draft, Writing – review & editing. **Daniel López García:** Methodology, Writing – review & editing, Project administration, Funding acquisition. **Enrique Giménez Torres:** Methodology, Validation, Writing – review & editing, Project administration, Funding acquisition. **Marta Fernández García:** Conceptualization, Methodology, Validation, Investigation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2022.111173.

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