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Communications

Topology Characterization by MALDI-ToF-MS of Enzymatically Synthesized Poly(lactide-*co*-glycolide)

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Lipase catalyzed copolymerization of the monomers lactide and glycolide by *Pseudomonas cepacia* employing a molar ratio of 80L/20G has been studied. The copolymers were characterized by MALDI-ToF-MS, DSC, SEC and NMR. MALDI-ToF-MS has successfully been used not only to determine end groups and chemical composition but even the microstructure of the copolymers. We demonstrated that for this lipase catalyzed copolymerization, the main product of the reaction at 100 °C was linear homopolymer of lactide while at 130 °C the main product was cyclic random copolymer.

Introduction

Homo and copolymers of lactide and glycolide have drawn increasing attention for medical applications due to their good biodegradability and biocompatibility. Especially, the use of poly(lactide-*co*-glycolide) as a matrix for drug delivery devices has grown rapidly over the past decades. Controlled drug release by controlled degradation of the polymer matrix is a topic in numerous publications.[1] Factors affecting the degradation characteristics are the copolymer's molar mass, molar mass distribution, and its chemical composition and microstructure. Knowledge of the exact topology of PLGA, which can attain a random, block, alternating, or gradient structure, is of high importance to govern degradation rates of the polymer matrix within the human body. The topology of the polymer is strongly determined by the synthetic route, which can be direct polycondensation of the hydroxyacids or copolymerization of lactide and glycolide (Scheme 1). Ring-opening polymerization can proceed anionically, cationically, and by a metal-catalyzed coordination/insertion mechanism. Especially, the latter method is widely employed due to the capability of performing stereospecific polymerizations resulting in high molecular weight polymer.

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As PLGA is predominantly used for medical applications, there is a strong interest in obtaining this polymer without the use of (toxic) metal catalysts. Enzymes might be a feasible solution to circumvent the use of these metal catalysts. Due to their capability for stereo-, regio-, and chemoselective polymerizations under mild reaction conditions, enzymes have gained increasing appreciation in the world of polymer synthesis. It was first reported by both the groups of Kobayashi[2] and Gutman[3] that lipases, enzymes capable of catalyzing the hydrolysis of fatty acid esters, can polymerize various medium-sized lactones, e.g., ϵ-caprolactone and δ-valerolactone. Nowadays, many different monomers have been polymerized by enzymatic ring opening such as cyclic anhydrides, carbonates, lactones, and lactides. Matsumura et al.[4,5] reported in 1997 the successful enzymatic polymerization of lactide using free and immobilized lipase PS.

Here, we report on the lipase-catalyzed copolymerization of the monomers D,L- or L-lactide and glycolide by *Pseudomonas cepacia*. Furthermore, the copolymer's topology was unraveled by using an in-house-developed method based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS). To the best of our knowledge, this is the first time that lipase-catalyzed copolymerization of lactide and glycolide has been reported.

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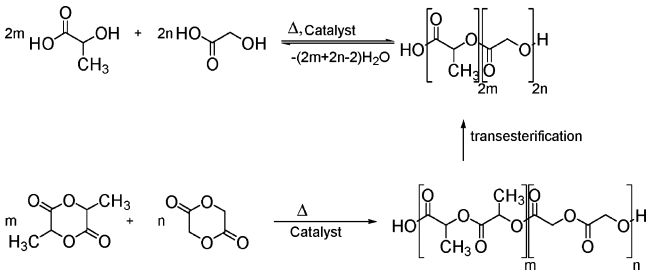
Scheme 1. Formation of Poly(lactide-*co*-glycolide) by Polycondensation of Lactic and Glycolic Acid and by Copolymerization of Lactide and Glycolide



Experimental Section

Reagents. D,L-Lactide (DLL) ((±)-3,6-dimethyl-1,4-dioxane-2,5-dione) and L-lactide (LL) ((3*S*)-*cis*-3,6-dimethyl-1,4-dioxane-2,5-dione) were purchased from Sigma Aldrich and glycolide (G) (1,4-dioxane-2,5-dione) from Avocado. Amano lipase PS (Sigma Aldrich) was used without further purification. Lipase PS is derived from *Burkholderia cepacia* and is also available immobilized on diatomaceous earth (lipase PS-D I). Glycolide and lipases were stored in a desiccator in the presence of P_2O_5.

Synthesis. A mixture of lipase PS (8 wt %), lactide (2.0 g, 13.9 mmol), and glycolide (0.4 g, 3.4 mmol) was reacted in an ampule placed in a thermostated oil bath at 100 or 130 °C under an argon atmosphere for 7 or 2 days, respectively. Next, the viscous mixture was dissolved in chloroform, and the enzyme was removed by filtration. The polymer was then precipitated by pouring the solution into methanol under continuous stirring. The precipitate was removed by filtration and dried in vacuo at 40 °C for 48 h.

NMR Analysis. NMR spectra were recorded on a Varian Mercury Vx (400 MHz) and a Varian Gemini 2000 (300 MHz) spectrometer. The solvents used were dimethyl sulfoxide-d_6 and chloroform-d_1.

DSC Analysis. The glass transition temperatures of the purified material were measured using a TA Instruments Q100 DSC equipped with a refrigerated cooling system (RCS) and autosampler. The DSC cell was purged with nitrogen gas flow of 50 mL min^{-1}. Experiments were performed in aluminum hermetic pans using a heating and cooling rate of 10 °C min^{-1}. The T_g was determined from the second heating curve by applying the half-extrapolated tangent method.

SEC Analysis. SEC analysis was carried out using a Waters model 510 pump, a model 410 refractive index detector (at 40 °C), and a model 486 UV detector (at 254 nm) in series. Injections were done by a Waters model WISP 712 autoinjector, using an injection volume of 50 μL. The columns used were a PLgel guard (5 μm particles) 50 × 7.5 mm column, followed by two PLgel mixed-C (5 μm particles) 300 × 7.5 mm columns at 40 °C in series. THF was used as eluent at a flow rate of 1.0 mL min^{-1}. For calibration, polystyrene standards were used (Polymer Laboratories, M_n = 580 to 7.1 × 10^6 g mol^{-1}). Data acquisition and processing were performed using Waters Millennium 32 (v 4.0) software.

MALDI-ToF-MS Analysis. MALDI-ToF-MS analysis was performed on a Voyager DE-STR from Applied Biosystems equipped with a 337 nm nitrogen laser. An accelerating voltage of 25 kV was applied. Mass spectra of 1000 shots were accumulated. The polymer samples were dissolved in THF at a concentration of 1 mg mL^{-1}. The cationization agents used were potassium trifluoroacetate (Fluka, >99%) or sodium trifluoroacetate (Fluka, >99%) dissolved in THF at a concentration of 1 mg mL^{-1}. The matrix used was *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) (Fluka) and was dissolved in THF at a concentration of 40 mg mL^{-1}. Solutions of matrix, salt, and polymer were mixed in a volume ratio of 10:1:5, respectively. The mixed solution was hand-spotted on a stainless steel MALDI target and left to dry. The spectra were recorded in the reflectron mode. Baseline corrections and data analyses were performed using Data Explorer version 4.0 from Applied Biosystems.

Table 1. Results of Lipase-Catalyzed Synthesis of PLGA

entry polymer[a]	enzyme	T_{rxn} (°C)	M_w[b] (kg mol^{-1})	PDI[b]	conversion[c] (%)	T_g[d] (°C)
1. PLLGA	PS	100	11.7	1.8	95	47.5
2. PLLGA	PS-D I	100	8.7	1.5	82	44.7
3. PLLGA		100	1.9	1.3	25	n.d.
4. PLLGA	PS	130	20.6	4.0	98	45.6
5. PLLGA	PS-D I	130	13.1	2.7	94	45.6
6. PLLGA		130	2.2	1.2	12	n.d.
7. PDLLGA	PS	130	13.5	3.2	94	43.7
8. PDLLGA	PS-D I	130	12.7	2.5	75	39.2

[a] Reaction time of 7 days for entries 1−3 and of 2 days for entries 4−8. [b] Determined by SEC. [c] Determined by integration of methine peaks in ^{1}H NMR. [d] Measured by DSC at a heating rate of 10 °C min^{-1}.

Results and Discussion

Enzymatic ring-opening polymerizations of lactide and glycolide (80L/20G) using both immobilized and free lipase PS were performed in bulk at 100 and 130 °C under an argon atmosphere. The copolymers were characterized by MALDI-ToF-MS, DSC, SEC, and NMR. The racemic mixture of lactide (D,L-lactide) was only used at 130 °C due to the higher melting point. The results are given in Table 1.

To be able to discriminate between enzymatically and nonenzymatically obtained polymers, reference reactions were performed in the absence of lipase. In the blank reaction at 100 °C, copolymer was formed with a molecular weight of 1.9 kg mol^{-1}, but monomer conversions were low in comparison to the enzyme-promoted reactions. Conversely, the blank reaction at 130 °C unexpectedly produced relatively high molecular weight polymer with high conversions of monomer after 7 days. A possible explanation for the polymerization in the blank reaction is the cationic polymerization by traces of hydroxyacid present in the monomers. Fortunately, even at 130 °C the nonenzymatic ring-opening polymerization is considerably slower than the enzymatic one and is left out of consideration for reaction times up to 2 days.

MALDI-ToF-MS spectra were analyzed using in-house-developed software to determine the end group, chemical composition, and topology of the polymer.[6,7] To assign a certain number combination of glycolyl and lactyl units to an experimental value of *m*/*z*, the program makes use of eq 1.

$$m_{cal} = n_G M_G + m_L M_L + E_I + E_{II} + M^+ \qquad (1)$$

E_I and E_{II} represent the molar masses of the end groups at opposite sides of the chain, $n_G M_G$ and $m_L M_L$ represent the number and mass of the repeating glycolyl and lactyl units, respectively, and M^+ the mass of the cation. A complete matrix with $n_{G,i}$ rows and $m_{L,j}$ columns can be constructed for a given end group using eq 1. Assignment of the peaks in the spectrum to a certain position in the matrix can be done employing an inequality

$$|m_{exp} - m_{cal}| \leq \frac{\Delta m}{2} \qquad (2)$$

in which m_{exp} represents the experimental mass, m_{cal} the calculated mass, and Δm the accuracy (1−2 g mol^{-1}). By calculating the natural abundance isotope distributions for each position in the matrix and rescaling it to the corresponding highest-intensity mass peak, a full spectrum can now be simulated.[8] Unfortunately, often multiple combinations of repeating units, $n_G M_G$ and $m_L M_L$, are possible for a certain value

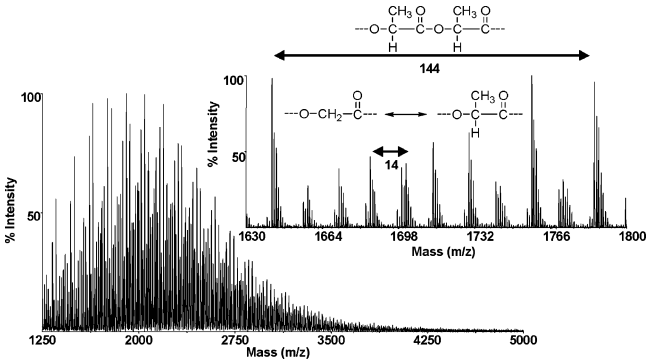


Figure 1. MALDI-ToF-MS spectrum and enlargement of PLLGA synthesized by lipase PS-D I at 100 °C (recorded with K^+).

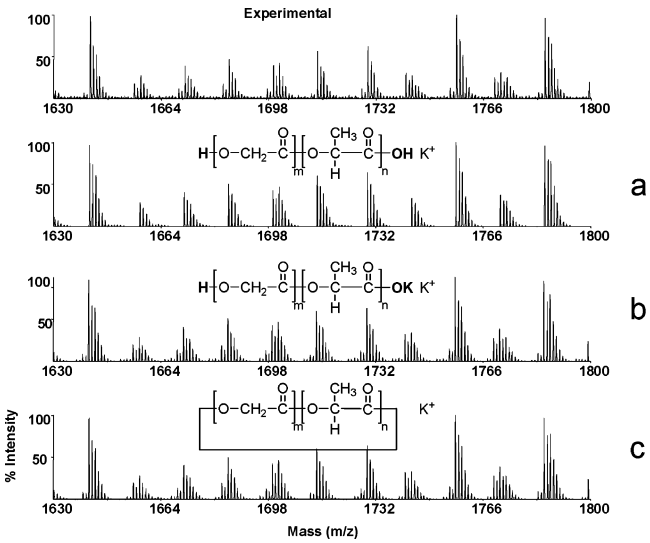


Figure 2. Experimental and simulated isotope patterns for different end groups of PLLGA synthesized by lipase PS-D I at 100 °C (recorded with K^+).

of *m*/*z* (multiple peak assignment), especially for higher molecular weight material. Moreover, the low difference in mass between lactyl and glycolyl units resulted in complicated, overlapping isotope patterns.

Standard MALDI-ToF-MS spectra were recorded in the reflectron mode using potassium trifluoroacetate as cationization agent. As can be seen in Figure 1, the difference between adjacent isotope distributions equals 14 *m*/*z* corresponding to the exchange of a glycolyl unit (58.01 g mol^{-1}) for a lactyl unit (72.02 g mol^{-1}). The difference between the most abundant peaks in the spectrum of PLLGA synthesized at 100 °C is 144 *m*/*z*, which corresponds to the mass of a lactydyl unit. This indicates the simultaneous presence of homopolymer polylactide (Figure 1).

Generally, polymer chains, obtained by water-initiated ring-opening polymerization of cyclic esters, are terminated by an alcohol and a carboxylic acid group (referred to as an H−OH end group). Occasionally, the acidic proton of the carboxylic acid end group can be replaced by a cation of the cationization agent, resulting in an H−OK end group. Finally, processes such as end-to-end condensation and/or intramolecular transesterification are responsible for the presence of cyclic structures. Simulation of the isotope distributions using the in-house-developed software resulted in Figure 2, in which Figure 2a,b,c are simulated spectra for H−OH, H−OK, and cyclic structures, respectively. Elimination of the presence of certain chain structures due to mismatching isotope patterns appeared impossible, since the most abundant distributions could be explained by all three possibilities.

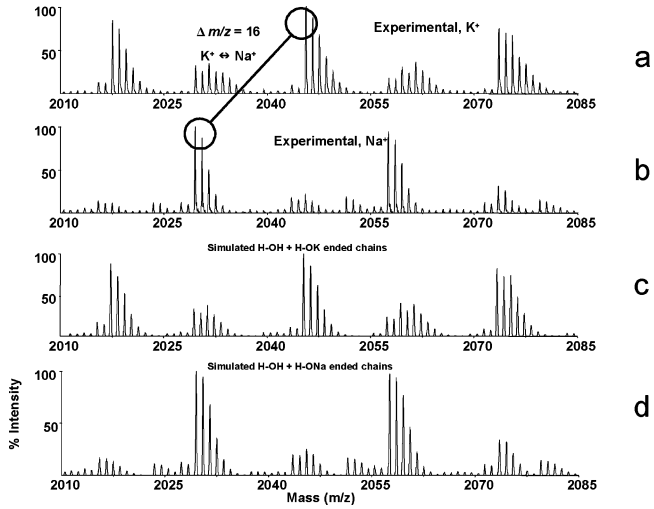


Figure 3. Experimental and simulated enlargement of the spectra for PLLGA (recorded with K^+ and Na^+). (Note: The low-intensity isotope distributions in the experimental spectrum cannot be explained by chains terminated by an H−OH end group. It was found that these distributions derive from a limited amount of chains where indeed the acidic proton is exchanged for a cation of the cationization agent (parts c and d).

Fortunately, end group determination can be facilitated by establishing the shift between the isotope distributions of spectra recorded with different cationization agents. Comparison of MALDI-ToF-MS spectra recorded with both potassium and sodium trifluoroacetate revealed a shift of −16 *m*/*z* between the most abundant peaks (Figure 3a and b). This automatically excludes the possibility that the highest-intensity peaks derive from H−OK (or H−ONa)-terminated chains. For these chains, a difference of 32 *m*/*z* should have been detected: the difference between an H−OK-terminated chain with a K^+ adduct and an H−ONa-terminated chain with a Na^+ adduct. This leaves H−OH end-capped and cyclic structures as the remaining possibilities for the highest intensity peaks.

To reveal the true structure of the polymer, we make use of a so-called contour plot, as these plots can provide information which cannot directly be derived from the MALDI-ToF-MS spectrum. The plots are based on the pioneering work of Wilczek-Vera et al.[9,10] The contour plot is a 2D representation of the normalized matrix of the mass spectrum (mentioned earlier) with lactyl and glycolyl units on the axes displaying the corresponding peak intensity of the combination (n_G, m_L). The position of the plot strongly depends on the end group, while the shape is hardly influenced by it. Information on the topology of the polymer is revealed by the shape and position of the plot. A line drawn through the optimum of the contour plot is a measure for the average chemical composition. If this line crosses the origin and the slope remains constant, the copolymer can be classified as random. When the line is curved but still crosses the origin, the plot represents a gradient copolymer. Finally, if the line does not cross the origin, we are dealing with a block copolymer.[6−8]

The presence of cyclic structures can be excluded on the basis of their contour plot (Figure 4a). The plot is abruptly cut off at the left of the column with red squares, which correspond to peaks of highest intensity in the MALDI-ToF-MS spectrum. Statistically, this cutting off is impossible due to equal chances of the syntheses for chains with one glycolyl unit more or less than the most abundant polymer chains, unless homopolymer

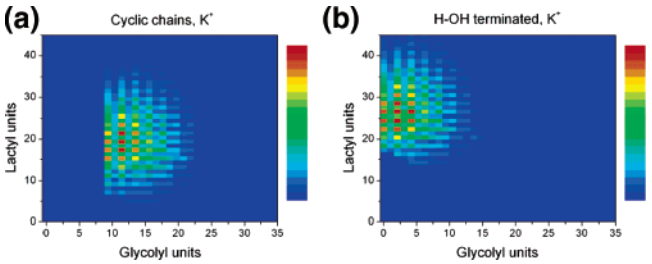


Figure 4. (a) Contour plot for cyclic structures. (b) Contour plot for H−OH-terminated chains. Both plots have been derived from the spectrum recorded with K^+ for PLLGA synthesized by lipase PS-D I at 100 °C.

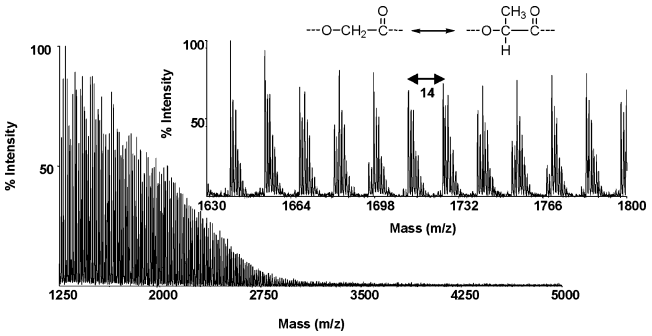


Figure 5. MALDI-ToF-MS spectrum and enlargement of PDLLGA synthesized by free lipase PS at 130 °C (recorded with K^+).

lactide is present. Then, subtraction of a glycolyl unit is impossible, as the number of glycolyl units cannot equal −1. A plot as Figure 4a strongly suggests that the end group is chosen wrongly and that the plot should in fact be positioned against the *y*-axis. This proved to be the case for chains terminated by an H−OH group (Figure 4b). Therefore, it can be concluded that the major part of the polymer chains is linear and H−OH end-capped.

Contour plots of copolymer synthesized by enzymatic ring-opening reactions performed at 100 °C show a curious phenomenon. Instead of the expected homogeneously colored area, the contour plot of PLLGA resembles a Scottish tartan pattern in which regularly distributed red squares can be seen (Figure 4). The first column of squares, counting from left to right, corresponds to the homopolymer of lactide, since no glycolyl units are present. Every second column shows the addition of a complete glycolydyl unit [-O−CH_2−C(O)−O−CH_2−C(O)-]. Similarly, every second row, a complete lactydyl unit is added to the polymeric chain. This indicates a dimer-wise incorporation of both monomers and negligible transesterification by lipase. Recently, we discovered a striped pattern in the contour plots of PLGA synthesized by $Sn(Oct)_2$, corresponding to the presence of chains odd- and even-numbered in glycolyl units but only even-numbered in lactyl units.[11]

The polymers synthesized by the lipase-catalyzed reaction performed at 130 °C were examined in a similar way as the polymers obtained at 100 °C. The isotope distributions have become equally intense with respect to the spectrum of PLGA at 100 °C, but the difference between the distributions remains 14 *m*/*z*, as expected. The MALDI-ToF-MS spectrum of PDLLGA, synthesized by free lipase PS, is given in Figure 5. We will omit here the procedure of comparison and simulation of spectra for PLLGA, because this does not deviate from PDLLGA synthesized at 130 °C.

Simulation of the possible end groups, similar to the polymerizations at 100 °C, resulted in the isotope patterns displayed in Figure 6. Surprisingly, while H−OH-terminated

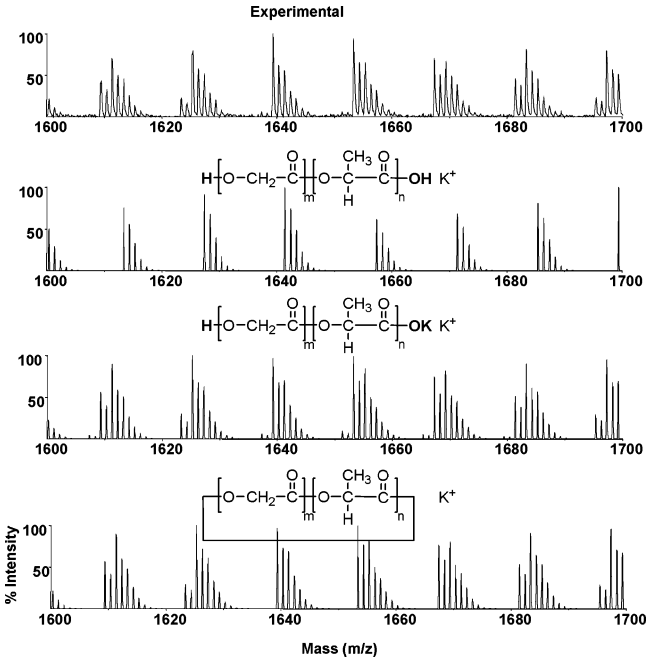


Figure 6. Experimental and simulated isotope patterns for different end groups of PDLLGA synthesized at 130 °C by free lipase PS (recorded with K^+).

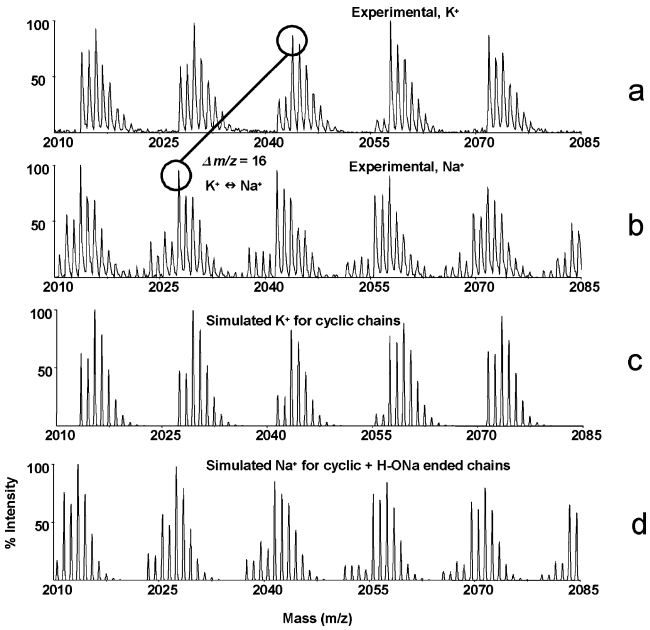


Figure 7. Experimental and simulated enlargement of the spectra for PDLLGA (recorded with K^+ and Na^+).

chains were the main polymer structures formed at 100 °C, at 130 °C the occurrence of the H−OH end group can be ruled out, simply because it cannot explain the isotope distributions present. Nevertheless, the simulations of cyclic structures and chains end-capped by H−OK again give rise to identical spectra.

Using potassium and sodium trifluoroacetate, a difference of 16 *m*/*z* is detected, ruling out the possibility of H−OK-terminated chains as mentioned before. In the spectrum measured with sodium, a few additional distributions are recorded in front of the most intense peaks which are absent in the potassium measured spectrum. These distributions are derived from polymer chains with an H−ONa end group. In Figure 7, the experimental and simulated isotope patterns clearly match.

In contrast to the polymer formed at 100 °C, the main structures in this case appear to be cyclic, as determined by

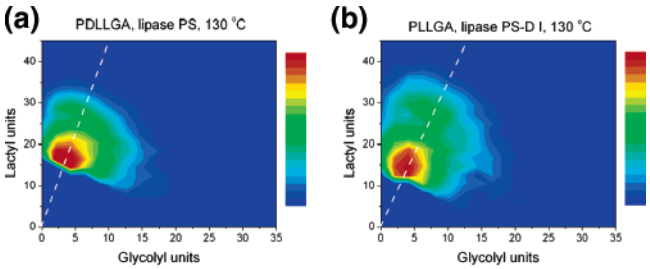


Figure 8. (a) Contour plot of PDLLGA synthesized by free lipase PS at 130 °C. (b) Contour plot of PLLGA synthesized by lipase PS-D I at 130 °C. (Both plots from spectra recorded with K^+.)

MALDI-ToF-MS. (Note: Differences in ionization efficiency limit MALDI-ToF-MS for quantitative applications. Therefore, other characterization techniques are required to determine the true ratio of cyclic to linear chains. Additionally, other measurements have shown that the ionization efficiency for cyclic structures differs for sodium and potassium cationization agents. Spectra recorded with potassium show more cycles than spectra recorded with sodium.) The contour plots for cyclic structures of PDLLGA and PLLGA synthesized by free and immobilized lipase, respectively, are shown in Figure 8. Interestingly, unlike the checkerboard pattern observed for polymer synthesized at 100 °C, the contour plots show a homogeneous area suggesting a highly increased (enzymatic) intermolecular transesterification. The most abundant polymer is the random copolymer of PLGA, as can be seen from the plot. Furthermore, no differences were observed between PLLGA and PDLLGA or between the free and immobilized lipase PS.

Conclusion

It is clear that the enzyme promotes the ring-opening copolymerization of lactide and glycolide because of the shorter reaction time and higher conversions of both monomers, but further investigation is needed to completely elucidate the mechanism and the role the enzyme is playing. Apparently, bulk polymerization of glycolide and lactide is possible at both 100 and 130 °C without the use of additional catalysts. The presence of a small amount of the hydroxyacids in the comonomers might be sufficient to initiate or even catalyze polymerization according to a cationic ring-opening polymerization mechanism. At this point, the enzymatic polymerization is not capable of competing with metal-catalyzed reactions from a molar mass and reaction time perspective.

Especially, when comonomers only differ by a methyl group, overlapping isotope distributions and multiple peak assignment are common which make the spectra and their simulation more complicated. Nevertheless, using intelligent software, more information can be derived from the experimental MALDI-ToF-MS spectrum than initially seemed possible, and this shows that MALDI-ToF-MS can be employed as a powerful tool to elucidate the polymer's fine structure and even its topology.

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Supporting Information Available. Additional information on evaluation methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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