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Journal:	*Biotechnology Journal*
Manuscript ID:	biot.201500074
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	16-Feb-2015
Complete List of Authors:	Pellis, Alessandro; University of natural Resources and Life Sciences Vienna, Department for Agrobiotechnology IFA-Tulln Herrero, Enrique; ACIB GmbH, Enzymes for polymers Weber, Hansjoerg; Graz University of Technology, Obersriebnig, Michael; University of Natural Resources and Life Sciences Vienna, Breinbauer, Rolf; Graz University of Technology, Institute for Organic Chemistry Srebotnik, Ewald; Vienna University of Technology, Guebitz, Georg; University of Natural Resources and Life Sciences, Vienna, Environment Biotechnology; ACIB GmbH, Enzymes for polymers
Primary Keywords:	Biocatalysis, Biomaterials, Analytical biotechnology
Secondary Keywords:	Biopolymers, Environmental biotechnology, Materials, Polymers
Additional Keywords:	poly(L-lactic acid), surface functionalization, enzymes
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Enzymatic functionalization of poly(L-lactic acid) films

Alessandro Pellis[a], Enrique Herrero Acero[b,*], Hansjoerg Weber[c], Michael Obersriebnig[d] Rolf Breinbauer[c], Ewald Srebotnik[e], Georg M. Guebitz[a,b]

[a] *University of Natural Resources and Life Sciences, Institute for Environmental Biotechnology, Konrad Lorenz Strasse 20, 3430 Tulln, Austria*

[b] *Austrian Centre of Industrial Biotechnology GmbH, Konrad Lorenz Strasse 20, 3430 Tulln, Austria*

[c] *Graz University of Technology, Institute of Organic Chemistry, Stremayrgasse 9, 8010 Graz, Austria*

[d] University of Natural Resources and Life Sciences, Institute of Wood Technology and Renewable Resources, *Konrad Lorenz Strasse 24, 3430 Tulln, Austria*

[e] *Vienna University of Technology, Institute of Chemical Engineering, Getreidemarkt 8, 1060 Vienna, Austria*

**Corresponding author: enrique.herreroacero@acib.at*

KEYWORDS: poly(L-lactic acid), surface functionalization, ^{14}C-Radiochemical analysis, enzymatic polymer functionalization, *Candida antarctica* lipase B

ABSTRACT

Poly(lactic acid) (PLA) as a biodegradable thermoplastic polyester has received increasing attention in the last years. This renewable polyester has found applications in a wide range of products such as food packaging, textiles and biomedical devices. Its major drawbacks are poor toughness, slow degradation rate and lack of reactive side-chain groups.

Here, an enzymatic process for the grafting of carboxylic acids onto the surface of poly(L-lactic acid) (PLLA) films was developed using *Candida antarctica* lipase B as a catalyst. Enzymatic hydrolysis of the PLLA film using *Humicola insolens* cutinase in order to increase the number of hydroxyl and carboxylic groups on the outer polymer chains for grafting was also assessed and showed a change in the water contact angle from 74.6° to 33.1° while the roughness and waviness were an order of magnitude higher in comparison to the blank.

The surface functionalization was demonstrated using two different techniques, ^{14}C-radiochemical analysis and X-ray photoelectron spectroscopy (XPS) using ^{14}C-butyric acid sodium salt and 4,4,4-trifluorobutyric acid as model molecules, respectively.

XPS analysis showed that 4,4,4-trifluorobutyric acid was enzymatically coupled based on an increase of the fluor content from 0.19% to 0.40%. The presented ^{14}C-radiochemical analyses are consistent with the XPS data indicating the potential of enzymatic functionalization in different reaction conditions.

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INTRODUCTION

Together with poly(caprolactone) (PCL), poly(lactic acid) (PLA) is one of the most widely studied polyesters due to its wide field of application and biodegradability.[1-3] PLA has excellent biocompatibility, biodegradability and processability properties[4-6] which makes it an attractive material for biomedical purposes.[7,8] The most common strategies to modify the bulk properties of PLA like mechanical characteristics include copolymerization with other monomers[9,10] and blending.[11-13] There is an increasing interest in modifying polymer surfaces in order to improve properties like hydrophilicity and/or creating reactive anchor groups for further functionalization. The latter includes for example covalent immobilization of bioactive compounds or decoration of nanoparticles for targeted drug delivery[14-16] while retaining the bulk properties.

Surface functionalization of polyesters is usually achieved via wet chemistry,[17-19] photografting[20-22] or plasma treatment.[23,24] In order to avoid the use of harsh chemicals and reduce the energy consumption different enzymes have been investigated to improve the surface hydrophilicity of renewable polyesters such as PLA[25-27] or even recalcitrant synthetic polyesters like poly(ethylene terephthalate) (PET).[28-30] Enzymes do not only work at mild process conditions and are not restricted to planar surfaces like plasma treatment but can specifically introduce modifications on polymer surface while leaving the bulk properties unchanged.

Improved biocompatibility of PLA, was achieved by coupling Human Serum albumin on cutinase activated membranes [31]. The functionalized material showed an increased antioxidant capacity as well as a higher osteoblast cell proliferation. In a similar approach, polyamide materials were functionalized in a two step enzymatic process starting with limited enzymatic surface hydrolysis.[32]

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The aim of the present work was to functionalize the surface of PLA films using hydrolases, both for surface “activation” and for the subsequent coupling of a model molecule. To monitor the surface functionalization process and avoid interferences due to adsorbed enzyme protein, a new method using ^{14}C-radiolabeled molecules was used for higher sensitivity. This enzymatic coupling approach is a promising environmentally friendly way to create PLA based materials where the surface is functionalized while leaving the bulk properties unchanged.

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MATERIALS AND METHODS

Chemicals and reagents.

L(-)-lactide was a gift from PURAC (Gorinchem, Netherlands). n-Butyric acid [1-^{14}C] sodium salt with a concentration of 1 mCi mL^{-1} in EtOH and a specific radioactivity of 50-60 mCi mmol^{-1} was purchased from American Radiolabeled Chemicals Inc. (St. Louis, USA). Poly(L-lactic acid) PLLA films, thickness 0.05 mm, were from Goodfellow (Cambridge, UK). All the other chemicals and solvents were purchased from Sigma-Aldrich at reagent grade, and used without further purification if not otherwise specified.

Enzymes.

Recombinant *Candida antarctica* lipase B (fCaLB) (62288 SIGMA) and CaLB immobilized on macroporous acrylic resin (L47770 SIGMA) (aCaLB) were from Sigma-Aldrich. *Humicola insolens* cutinase (HiC) was a gift from Novozymes (Beijing, China) and was purified as previously described[33] prior to using. Covalently immobilized CaLB (cCaLB) was kindly provided by SPRIN S.p.a. (Trieste, Italy).

Esterase activity assay.

Esterase activity was measured at 21˚C using p-nitrophenyl butyrate (PNPB) as a substrate as previously reported by Ribitsch et al. with some modification.[26] The final assay mixture was made up of 10 µL of the substrate solution (86 µL of PNPB in 1000 µL of DMSO), 1000 µL of 100 mM Tris-HCl buffer at pH 7 and 100 µL of enzyme solution. The increase of the absorbance at 405 nm due to the hydrolytic release of p-nitrophenol (ε 405 nm= 9.36 mL (µmol cm)$^{-1}$) was measured over time using a Jasco V-630 spectrophotometer using plastic cuvettes. A blank was included using 100 µL of buffer instead of enzyme. The activity was calculated in units (U), where 1 U is defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay conditions.

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Synthetic activity assay.

Quantification of the synthetic enzyme activity was performed based on the enzymatic synthesis of propyl laurate. Briefly, 7.2 g of 1-propanol and 24 g of lauric acid were weighted into a 100 mL glass bottle. The solution was heated up to 60 ˚C until the lauric acid was completely dissolved. A sample at time= 0 min was taken and the biocatalyst was added, while maintaining the solution under continuous stirring. Samples were withdrawn at different reaction times. The assay is based on the determination of acid values of the reaction mixture by titration with 0.1 M KOH in ethanol using phenolphthalein as indicator. The acid value indicates the residual unreacted lauric acid. One unit of enzyme activity is defined as the amount of enzyme protein required to produce 1 µmol of propyl laurate per minute under standard conditions (T=60 ˚C).

Protein quantification.

Protein concentration was determined with the BioRad protein assay (Bio-Rad Laboratories GmbH, Vienna, Cat.No: 500-0006). Briefly, 10 µL of the sample was added into the wells of a 96 well micro-titer plate. As soon as all the samples were in the wells, 200 µL of the prepared BioRad reaction solution were added (BioRad Reagent diluted 1:5 with Fresenius water). The plate was incubated for 5 min at 21 ˚C and 400 rpm. Buffer (0.1 M Tris-HCl pH 7) was used as blank and BSA as standard. The absorption after 5 min was measured at λ=595 nm and the concentration calculated from the average of triplicates.

PLLA Hydrolysis.

Prior to the treatment, PLLA films were cut into pieces (20×20 mm) and washed in three consecutive steps for 30 min at 37 ˚C and 130 rpm. In a first step, a solution of 5 g L^{-1} Triton-X100 was used, in the second step a 100 mM Na_2CO_3 solution was used, followed by double distilled water (ddH_2O). The *Humicola insolens* cutinase was diluted in 100 mM Tris-HCl buffer pH 7 and used in different concentrations. Chemical hydrolysis was

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performed using NaOH in concentrations from 0.05-0.1 M. Incubations were performed at 130 rpm and 37 ˚C for the enzymatic treatment and at 21 ˚C for the alkaline treatment. Hydrolysis products were quantified by using high performance liquid chromatography. Controls were performed by incubating the PLLA with buffer solution.

PLLA functionalization.

After the hydrolytic treatment, PLLA films were incubated in a 0.2 M butyric acid solution in anhydrous n-heptane containing different concentrations of n-butyric acid [1-^{14}C] sodium salt. Different preparations and amounts (calculated in U g^{-1}) of *Candida antarctica* lipase B as biocatalyst were used. For the X-ray photoelectron spectroscopy (XPS) measurements the functionalization was carried out using a 0.2 M 4,4,4-trifluorobutyric acid solution in n-heptane as above.

Synthesis of PLLA oligomers.

The preparation of the PLLA oligomers was carried out as previously described[34] with some modification.

Preparation of (*S*)-2-((*S*)-2-hydroxypropanoyloxy)propanoic acid (oLACT).

25.003 g of (3*S*,6*S*)-3,6-dimethyl-1,4-dioxane-2,5-dione in water (150 mL) were stirred at 45 ˚C for 4 h. The reaction mixture was then freeze dried to obtain the product as a clear colorless oil.

Synthesis of benzyl 2-((*S*)-2-hydroxypropanoyl)oxy)propanoate (pLACT).

24.008 g of oLACT, 43.350 g of benzyl bromide, 50.608 g of triethylamine and 250 mL of CH_2Cl_2 were put in a 500 mL glass bottle under ice. Triethylamine and the CH_2Cl_2 were later removed under reduced pressure. The subsequent workup of the reaction was performed according to the method cited above.

Enzymatic synthesis of 1-((1-(benzyloxy)-1-oxopropan-2-yl)oxy)-1-oxopropan-2-yl butyrate (pLACT-BA).

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For the synthesis of pLACT-BA 0.2049 g (0.81 mmol) of pLACT and 0.0718 g (80.81 mmol) of butyric acid (BA) were put in a 4 mL glass vial with 10% w w^{-1} immobilized CaLB as catalyst under nitrogen atmosphere. The reaction mixture was then stirred for 96 h at 100 ˚C. A control reaction was carried out at the same conditions without catalyst. Detailed spectral information of the model substrates can be found in the supplementary information.

HPLC-RI.

Hydrolysis samples were precipitated following the Carrez method and filtered through 0.20 µm Nylon filters (GVS, Indianapolis, USA). The analytes were separated by high performance liquid chromatography (HPLC) using refractive index detection (1100 series, Agilent Technologies, Palo Alto, CA) equipped with an ICSep-ION-300 column (Transgenomic Organic, San Jose, CA) of 300 mm by 7.8 mm and 7 µm particle diameter. Column temperature was maintained at 45 °C. Samples (40 µL) were injected and separated by isocratic elution for 40 min at 0.325 mL min^{-1} in 0.005 M H_2SO_4 as the mobile phase.

GPC.

Samples were dissolved in THF (250 ppm BHT as inhibitor) and filtered through filter paper (595 ½, Whatman GmbH, Dassel, Germany). In case of liquid samples, the starting solvent was removed under reduced pressure. Gel permeation chromatography was carried out at 30 °C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17369 6.0 mm ID x 40 mm L H_{HR}-H, 5 µm Guard column and a 18055 7.8 mm ID x 300 mm L GMH_{HR}-N, 5 µm TSKgel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using THF (250 ppm BHT as inhibitor) as eluent (at a flow rate of 1 mL min^{-1}). An Agilent Technologies G1362A refractive index detector was employed for detection. The molecular weights of the polymers were calculated using linear polystyrene calibration standards (400-2000000 Da).

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Hydrophobicity measurements.

Hydrophobicity of the sample was measured via Water Contact Angle (WCA). WCA of the PLLA films were measured before and after exposure to the enzymatic treatments. The protein was washed away from the surface using three consecutive washing steps [26, 28] as described in the PLLA hydrolysis section. Polymer films were analyzed with a Drop Shape Analysis System DSA 100 (Kruss GmbH, Hamburg, Germany) using ddH_2O as test liquid with a drop size of 2 µL, deposition speed 100 µL min^{-1}. Water contact angles were measured after 5 s and data are obtained from the averages of the measurements taken from at least eight different points of three sample surfaces.

Atomic Force Microscopy (AFM) Microscopy.

All AFM-microscopy was performed on a Bruker Dimension Icon AFM (Bruker, Karlsruhe, Germany) in tapping mode. The probes used were OTESPA (AFM Bruker, Karlsruhe, Germany) and had nominal values of k= 42 N m^{-1}, f_0= 300 kHz and r_{tip} <10 nm. Scan speed was 1 Hz, with gain factors and set point kept as constant as possible with only small adjustments to get good feedback. Scan size for all images was (1 µm)^2, with a resolution of 512 pixels $line^{-1}$ for the reference PLLA and the non-pretreated samples. A resolution of 256 pixels $line^{-1}$ was used for the pre-hydrolyzed samples. This change was performed to lower the effect of uncontrolled oscillations of the cantilever on these samples.

Evaluation of AFM images was performed using the free Gwyddion 2.28 image analysis software. For all images a tilt correction on the topography image was performed and the minimal value set to zero before data analysis. Roughness analysis evaluating r_a, r_q, w_a and w_q, was performed by evaluating the length of the image diagonals. The width of the analysis strip was 5 pixels (256 pixels $line^{-1}$ resolution images) and 10 pixels (512 pixels $line^{-1}$ image resolution) respectively, with a roughness cut-off of 0.1 for all images.

^{14}C-radiochemical analysis.

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The functionalized films were put into plastic vials containing 10 mL scintillation cocktail (Optiphase Hisafe 3, Perkin-Elmer) and assayed for ^{14}C on a Perkin-Elmer Tri-Carb 3110 liquid scintillation counter (dpm mode, 20 min counting time). Liquid samples (100 µL) were mixed with scintillation cocktail (2 mL) and assayed for ^{14}C as described above.

X-ray photoelectron spectroscopy (XPS).

XPS was performed on an Multiprobe UHV-surface-analysis system (Omicron Nanotechnology) equipped with a DAR400 x-ray source with Al anode and a quartz-crystal monochromator XM 500 using a x-ray excitation energy of 1486.70 eV (Al Kα1-line). The monochromated x-ray line width was of 0.3 eV. The analyzed surface area was of approximately 1 mm^2. The reaction mixture was then stirred for 25 h at 21 ˚C and monitored via TLC.

Lyophilization.

Freeze drying was conducted using a Christ Freeze dryer Beta 1-16, 220 V, 50 Hz, 1.2 kW. 1-dimensional roughness and waviness of the graphs was evaluated from line spectra of the image diagonals as r_q and w_q, that is the root mean square height deviation of the mean line. The cut-off frequency was chosen as 0.1.

Flash Column Chromatography.

For the purification of pLACT a Büchi flash column chromatography system equipped with a C-620 Control Unit, a C-660 Fraction Collector and a C-640 UV spectrophotometer (Büchi, Switzerland) was used. Sepacore® HP 120 g Büchi Silica cartridges were used as stationary phase and a mixture of 1:1 hexane:ethyl acetate was used as mobile phase for separations.

TLC.

Analytical thin layer chromatography (TLC) was performed on commercial Macherey-Nagel plates coated with silica gel Alugram® SIL G/UV_{254} (0.2 mm thick using hexane/ethyl

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acetate in a ratio of 1:1 as the mobile phase. The analytes were detected using a UV lamp or via staining with a $KMnO_4$/KOH solution.

LC/TOF-MS.

Samples were dissolved in acetonitrile (AcN) with 0.1% formic acid at a concentration of 1 mg mL^{-1} and filtered through 0.20 µm Nylon filters.

Liquid chromatography/electrospray/time-of-flight mass spectrometry (LC/ESI/TOF/MS), in positive ionization was used to separate and identify oLACT and pLACT. The analytes were separated using an HPLC (1260 series, Agilent Technologies, Palo Alto, CA) equipped with a reversed-phase C18 rapid resolution column (Zorbax Eclipse XDB, Agilent Technologies) of 50 mm by 2.1 mm and 1.8 µm particle diameter. Column temperature was 25 °C. Mobile phase A was water with 0.1% formic acid, and mobile phase B consisted of AcN with 0.1% formic acid. The mobile phase composition was maintained at the initial conditions (90% A) for 0.5 min, followed by a linear gradient progressed to 100% B in 35 min, after which the mobile phase composition for 5 min. The next 10 minutes mobile phase was set back to the initial conditions. The flow rate was 0.4 mL min^{-1} and the injection volume was 1 µL. This HPLC system was connected to a time-of-flight mass spectrometer (6230 TOF LC/MS, Agilent Technologies) equipped with an electrospray interface under the following operating parameters: capillary 3500 V, nebulizer 20 psig, drying gas 10 L min^{-1}, gas temperature 325 °C, fragmentator 200 V, skimmer 65 V, OCT 1 RF Vpp 750 V. The mass axis was calibrated using the mixture provided by the manufacturer over the *m*/*z* 50-3200 range. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: 121.050873 and 922.009798 *m*/*z*. Spectra were acquired over the *m*/*z* 100-3000 range at a scan rate of 2 spectra s^{-1}.

NMR.

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Nuclear magnetic resonance ^{1}H and ^{13}C measurements were performed on a Bruker Avance II 400 spectrometer (resonance frequencies 400.13 MHz for ^{1}H and 100.63 MHz for ^{13}C) equipped with a 5 mm observe broadband probe head with z–gradients. $CDCl_3$ was used as NMR solvent if not otherwise specified.

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Results and discussion.

The biodegradable and biobased polyester PLLA is increasingly being used in many fields from textiles to biomedical usually involving chemical functionalization. Current covalent PLLA surface-modification methods usually involve the use of UV irradiation for the grafting of molecules onto the PLLA surface. However, this technique leads to monomer migration into the film bulk[5] and a noticeable decrease of its molecular weight after irradiation.[35] In this work we describe a novel non-destructive enzymatic method for surface functionalization of PLLA films that prevents bulk modification of the polyester and at the same time allows a controlled grafting onto the polymer surface. In a first step new reactive groups are created onto the polymer surface by limited enzymatic hydrolysis of the outer PLLA polyester layers, followed by a subsequent lipase-catalyzed coupling of a molecule containing carboxylic functionalities onto the PLLA surface.

PLLA hydrolysis.

In a first step, enzymatic surface hydrolysis of PLLA was studied. Therefore, the release of lactic acid as quantified with HPLC-RI (Figure 1) was compared for changes in bulk properties as measured by using GPC. After 24 h of hydrolysis, a maximum of 42 mg L^{-1} of lactic acid was released. Longer incubation times of 72 h were also assessed leading to a release of 113 mg L^{-1} of lactic acid. No release of lactic acid was detected in control conditions. The results from GPC measurements showed that there were only slightly changes in M_w, M_n and polydispersity indices of the PLLA after 72 h of treatment (Table 1). This indicates, that the hydrolysis preferentially occurred on the polymer surface while the bulk properties were not significantly altered in contrast to chemical alkaline hydrolysis.

Chemical PLLA hydrolysis such as alkaline hydrolysis, can lead to unexpected results on the degradation rate depending on the polymer sources.[36] As alkali-based treatments strongly affect the bulk properties of the PLLA films, this technique is not suitable if the

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final goal is to achieve only a surface-functionalization of the polymer. In addition, the residual alkali is not easily removed after the treatment.[9]

Limited partial hydrolysis of the PLLA as performed here generates hydroxyl and carboxylic groups leading to hydrophilization of the surface. Therefore, water contact angle (WCA) measurements after complete enzyme removal were used to quantify the hydrophobicity decrease upon enzymatic surface hydrolysis. Compared to the blank (74.6˚±0.7˚), the maximum hydrophilicity of the WCA was reached already after 24 h of hydrolysis (33.1˚±1.7˚) with a marginal further decrease after longer incubation for 48 h (33.7˚±2.1˚) and 72 h (36.5˚±0.9˚). The WCA value of the starting PLLA film was 73.5˚±1.2˚. Previously, slightly higher values were obtained for enzymatic hydrolysis of PLLA membranes which are most likely due to a larger surface area.[31] Nevertheless, these results indicated that 24 h surface hydrolysis should be enough to activate the PLLA for further functionalization. At those particular conditions describer by Nyanhongo et al. the hydrophilicity increase achieved with the enzymatic treatment, 58˚±3˚, was comparable to the one achieved with a chemical treatment 52˚±5˚ (hydrophobicity of the starting material: 78˚±2˚).[31]

AFM analysis of the enzymatically hydrolyzed samples revealed distinct morphology changes created on the surface (Figure 2).

Compared with the untreated PLLA (Figure 2A), which shows a nearly ideally smooth surface, with small evenly scattered structures, the enzymatically hydrolyzed samples (Figures 2B, D and F) showed a rougher surface. Roughness and waviness (Table S1) were an order of magnitude higher compared to the blank (Figures 2C and E), with valley-to-peak distances of about 500 nm on a scan length of only 1000 nm.

Collectively, these results strongly indicate that carboxylic and hydroxyl groups can be generated enzymatically on PLLA films using the cutinase from *Humicola insolens* with low extent of damage to the material. WCA measurements showed that the treatment leads to

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a strong decrease of the surface hydrophobicity (more than 40˚ after 24 h of treatment in comparison with the blank) due to the new carboxyl and hydroxyl generated groups without affecting the bulk structure of the polymeric material. The same degree of modification (as measured with WCA) is not possible by using alkali treatment without a concomitant strong decrease of the PLLA molecular weight.[37] Controlled surface hydrolysis of PLLA for activation of the material for further functionalization is investigated in this study. Moreover, hydrophilization per se is required for certain applications such as to improve cell affinity.[38]

Enzymatic esterification

In a first step, enzymatic esterification of PLLA was studied using oligomers as PLLA models. Therefore, a -COOH benzyl protected lactide dimer (pLACT) was successfully synthesized in order to obtain a PLLA dimer with only one reactive end (-OH group). The esterification of pLACT with butyric acid was then carried out using *Candida antarctica* lipase B (CaLB) adsorbed onto macroporous acrylic resin as catalyst. CaLB is able to catalyze the esterification reaction of various dicarboxylic acids with diols in different solvents[39,40] and is also capable to trans-esterify aromatic dicarboxylic acid esters with diols.[41]

The synthetic route that leads to pLACT coupled with butyric acid (pLACT-BA) is shown in Scheme 1. The compounds were used in a 1:1 ratio in bulk.

In order to demonstrate that CaLB is able to catalyze the esterification of the PLLA model pLACT as shown in Scheme 1, reaction mixtures were analyzed by LC/TOF-MS. Despite the occurrence of some uncatalyzed background conversion,[42] the difference between blank and enzymatic reaction appeared to be highly significant. Since an absolute quantification of the reaction product with LC/MS-TOF analysis is not possible due to the presence of Na^+ adducts, we calculated the difference between blank and enzymatic treatment according from the UV spectra of the HPLC peaks identified as pLACT and

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pLACT-BA by TOF-MS. A pLACT-BA/pLACT ratio of 0.02 at λ = 260 nm (absorption maximum of the benzyl group) was found for the blank while a pLACT-BA/pLACT ratio of 0.46 was found for the CaLB catalyzed reaction. The model reaction thus demonstrated that CaLB is a suitable catalyst for the esterification reaction between the protected dimer pLACT with a carboxyl containing molecule (butyric acid). The reaction was also performed using n-heptane as solvent and showed similar results (pLACT-BA/pLACT ratio for the enzymatic reaction of 0.34, pLACT-BA/pLACT ratio for the blank reaction of 0.01).

Functionalization of PLLA films.

After proven that CaLB is able to catalyze the esterification of PLLA oligomers, in a next step the lipase enzymatic esterification of PLLA films making use of the carboxylic and hydroxyl groups enzymatically generated on the film surface was investigated.

Since enzymatically activated PLLA films were not soluble in butyric acid the reaction was carried out in an organic solvent as previously reported for other polyesters.[17,43] Since PLLA is known to be soluble in most of the chlorinated and aromatic organic solvents,[44] the first step involved identification of an appropriate solvent system not dissolving the PLLA film while being suitable for the enzymatic synthesis. n-Heptane was found to fit both requirements: non-solubility of the PLLA film and possibility of conducing a esterification reaction using CaLB as catalyst. Incubation of n-heptane with the polymer film revealed no dissolution or degradation of the polymer after up to 72 h of incubation both with or without prior enzymatic activation.

After solvent selection, the next challenge was to develop a sensitive method to detect the binding of the molecule on the polymer surface during optimization of reaction conditions. Commonly techniques like ATR-FTIR, SEM and hydrophobicity measurements were found to be unable detect the coupled molecule. ATR-FTIR is usually a very powerful technique to detect surface functionalization of polymers[17,43] but IR-light is penetrating into the polymer bulk, making the analysis of the outer layers of the polymer involved in the above

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described functionalization unfeasible.[35] SEM was used for the study of several polylactides[38,45] but for our system it turned out to be a destructive technique. Hydrophobicity measurements, really helpful in the study of the hydrolysis of several polyesters,[26,28] were not suitable to detect the newly bound molecules on the polymer surface because of the limited amount of molecule that was coupled via the enzymatic treatment. Therefore, ^{14}C-radiolabeled butyric acid was used for esterification together with ^{14}C-radiochemical analysis of the treated film.

Both the pre-hydrolyzed and the non-pretreated PLLA films, showed consistently higher scintillation counts in presence of CaLB compared to the PLLA samples that had not undergone the enzymatic treatment. In all cases some background sorption of the radiolabeled molecule was detected. Sorption studies indicated that the amount of butyric acid in the initial reaction solution determines the base level of sorption of butyric acid on the film and a direct correlation between the amount adsorbed and the initial radiolabeled butyric acid concentration was clearly seen. In the presence of the enzyme, a clear increase on the amount of butyric acid over time on the PLLA was observed (Figure 3).

Interestingly, both the pre-hydrolyzed and the non-pretreated samples show similar levels of functionalization. This may be due to the fact that in an apolar reaction environment such as n-heptane, the newly generated polar surface groups (-OH and -COOH), undergo hydrophobicity recovery. This recovery in air has already been widely described for polymers modified via corona discharges or plasma.[46] Previous studies demonstrated that the hydrophilic surface is unstable and that there is a rearrangement of the polymer chains to a more thermodynamically stable state. Based on the above mentioned findings, we hypothesize a polymer reorientation also for our PLLA/n-heptane reaction system. The cleaved surface chains are turning inside the polymer matrix, with the uncleaved chains (such as those found in the non-hydrolyzed sample) becoming exposed to the enzyme for the coupling reaction.

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This effect was investigated by conducting hydrophobicity measurements after the partial hydrolytic treatment and incubation of the samples for 24 h in n-heptane. The solvent was removed incubating the samples at 30 °C for 24 h under a continuous air flow. Water contact angle measurements (WCA) revealed indeed a hydrophobicity recovery during the coupling reaction, in which the WCA after enzymatic hydrolysis increased from 33.1˚±1.7˚ to 53.3˚±2.82˚ after incubation for 24 h in n-heptane. These data clearly confirm that the PLLA films undergo a significant surface hydrophobicity recovery when incubated in apolar solvents explaining the similar relativities of pre-hydrolyzed and non-pretreated samples.

It is important to note the effect of the amount of dosed enzyme on the degree of esterification achieved. Using 880 U of aCaLB, the amount of dpm film^{-1} detected was 15.5±1.3 counts for the non-pretreated sample and 13.8±1.9 counts for the pre-hydrolyzed sample; while using 88 U of enzyme, the amounts of dpm film^{-1} detected were 9.0±0.8 and 7.3±0.5 counts, respectively. Both experiments were conducted using the same concentration of butyric acid (0.2 M) with a ^{14}C-radiolabeled substrate of 35 kdpm mL^{-1}, which lead to a background butyric acid sorption of 6.5±2.4 counts for the non-pretreated sample and 7.5±2.6 for the pre-hydrolyzed sample (Table S2).

The clear influence of the enzyme dosage on the second step in the polymer functionalization, led us to investigate whether the type of CaLB preparation would affect the esterification yield. Therefore we performed experiments comparing lyophilized CaLB (fCaLB) with covalently immobilized CaLB (cCaLB). In the first case, the enzymatic treatment (400 U) showed a consistent effect (22.7±2.1 dpm film^{-1} for non-pretreated PLLA, 23.3±1.0 dpm film^{-1} for pre-hydrolyzed PLLA) in comparison with the blank (11.0±0.8 dpm film^{-1} for non-pretreated PLLA, 13.3±0.5 dpm film^{-1} for pre-hydrolyzed PLLA). The functionalization was only seen when using at least 400 U of fCaLB. These experiments were conducted using a starting concentration of ^{14}C-radiolabeled substrate of 42 kdpm mL^{-1}. In the case of cCaLB (860 U, starting concentration of ^{14}C-radiolabeled

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substrate of 58 kdpm mL^{-1}), a comparable esterification after 24 h as previously described for the adsorbed enzymatic preparation (aCaLB) was observed (Table S3).

Interestingly, there were no significant differences in terms of final butyric acid content regardless what the first step was. Especially remarkable is the fact that the enzymatic generation of hydroxyl groups did not increase the amount of butyric acid bound on the polymer film after incubation with neither immobilized nor free CaLB. However, independently from the first step a significant increase of the amount of butyric acid grafted was detected with any of the used CaLB preparations.

The surface composition of the enzymatically functionalized films was further characterized by XPS[47] using fluorine "labeled" butyric acid. The XPS spectra indicate that both the CaLB treated and untreated samples contain fluorine atoms and consequently fluoro butyric acid on the surface while the starting PLLA film and the same samples after 24 h of hydrolysis only contain carbon and oxygen (H excluded). Nevertheless, despite some adsorption, there was a clear increase in the amount of fluoro butyric acid from 0.19% to 0.40% upon enzymatic esterification. These data are therefore consistent with ^{14}C-radiochemical analysis and provide an additional means of quantification of the amount of substrate coupled to the surface of the PLLA film.

Overall the ^{14}C-radiochemical method proved to be very sensitive and allowed the detection of even small amounts of butyric acid enzymatically grafted onto the PLLA surface. This technique is not influenced by enzyme potentially adsorbed and not-completely removed from the film prior to analysis. This has been reported to produce artifacts in XPS analysis for monitoring enzymatic surface hydrolysis of PET.[48,49] However, in this study we have circumvented this problem by using a substrate "labeled" with fluorine which is neither present in the polymer nor in the enzyme preparation.[50,51]

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CONCLUSIONS

An innovative enzymatic method for the surface functionalization of poly(L-lactic acid) films is presented. PLLA films were functionalized with ^{14}C-radio labeled butyric acid or 4,4,4-trifluorobutyric acid using *Candida antarctica* lipase B as catalyst in n-heptane at a reaction temperature below the glass transition temperature ($T<T_g$) of the biopolymer. The feasibility of the functionalization was investigated via ^{14}C-radiochemical analysis while the surface composition was investigated via XPS analysis. No significant difference in the yield was observed while functionalizing pre-hydrolyzed and non-pre-hydrolyzed polymer. This is most probably due to a rearrangement of the outer polymer chains in the hydrophobic reaction environment. It is indeed remarkable how PLLA films maintained their bulk properties despite the enzymatic functionalization of the surface in contrast to other commonly used surface modification methods previously reported so far. The results presented here form the basis to further investigate enzymatic functionalization to tune PLLA properties using more complex substrates like those of biomedical interest.

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Acknowledgments.

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement N° 289253.

Notes.

The authors declare no competing financial interest.

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Table 1. Number-average molecular weights and polydispersity indices of the PLLA films before and after the enzymatic surface hydrolysis.

Treatment	M_n (g mol^{-1})[a]	M_w M_n^{-1}[a]
Starting PLLA	$34.6*10^3$	4.8
Blank 24 h	$34.3*10^3$	4.8
Blank 48 h	$35.3*10^3$	4.7
Blank 72 h	$34.0*10^3$	4.8
Hydrolysis 24 h	$34.6*10^3$	5.0
Hydrolysis 48 h	$33.3*10^3$	5.0
Hydrolysis 72 h	$34.3*10^3$	5.0

[a]Determined using GPC calibrated with narrow molar mass polystyrene standards.

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Figure 1. Lactic acid released from poly(L-lactic acid) films hydrolyzed during hydrolysis by *Humicola insolens* cutinase (HiC) in 0.1 M TRIS buffer at pH 7. Black: 1.0 mg mL^{-1} HiC; dark grey: 0.2 mg mL^{-1} HiC; gray: 0.1 mg mL^{-1} HiC. Control reactions performed using buffer solution led to no detectable hydrolysis products.

Figure 2. Atomic force microscopy phase images of the surfaces of PLLA films after enzymatic functionalization with and without enzymatic prehydrolysis. (A) PLLA blank (24 h in buffer), (B) 24 h hydrolyzed with cutinase, (C) Non-hydrolyzed/enzymatic coupling blank, (D) Hydrolyzed/enzymatic coupling blank, (E) Non-hydrolyzed/enzymatic coupling, (F) Hydrolyzed/enzymatic coupling. All AFM pictures were scanned over an area of 1 µm x 1 µm.

Figure 3. CaLB catalyzed functionalization of PLLA films with butyric acid with and without prior enzymatic surface hydrolysis by cutinase HiC. Light grey: CTRL-CTRL; grey: HiC-CTRL; dark grey: CTRL-CaLB; black: HiC-CaLB. The experiments were conducted using a starting concentration of ^{14}C-radiolabeled butyric acid of 58 kdpm mL^{-1}.

Scheme 1. Reaction scheme of the pLACT-BA synthesis. A: Ring opening of L-lactide in order to obtain oLACT. B: Protection of the -COOH end group of the oLACT using benzyl bromide (BB) in order to obtain the protected dimer with only one functional end (pLACT). C: Enzymatic esterification of pLACT with butyric acid (BA) using CaLB as biocatalyst (reaction performed both in bulk and in n-heptane as reaction solvent).

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Supplementary material

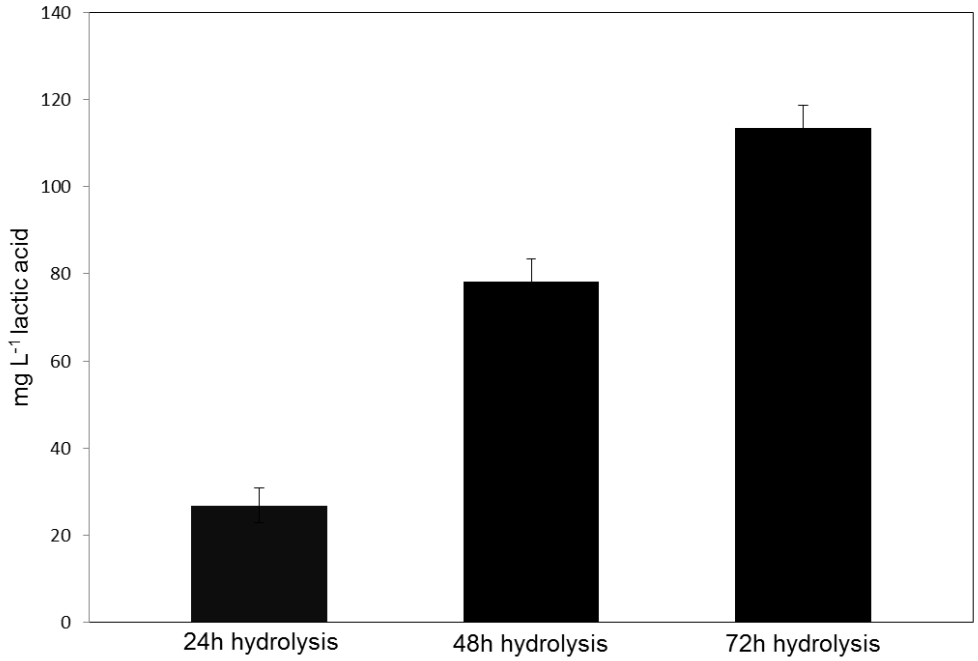

Figure 1. Released products (LA) during long-term hydrolysis study of the poly(L-lactic acid) films hydrolyzed by 0.1 mg/mL *Humicola insolens* cutinase (HiC) in 0.1 M TRIS buffer at pH 7. Control reactions performed using buffer solution led to no detectable hydrolysis product up to 72 h of incubation.

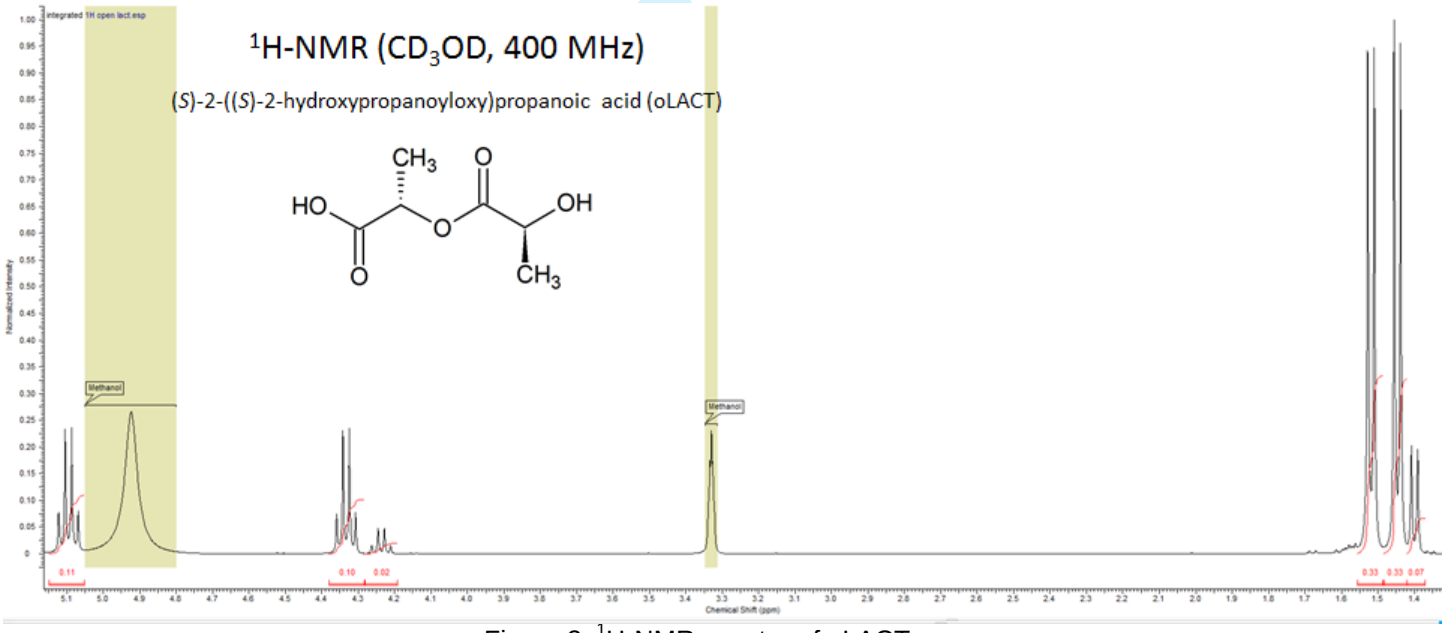

Figure 2. ^{1}H-NMR spectra of oLACT.

Spectral information:

^{1}H-NMR (MeOD-d_4): δ 5.07 (q, J= 7.1 Hz, $CO_2C\mathbf{H}(CH_3)CO_2H$, 1H), 4.31 (q, J= 6.9 Hz, $HOC\mathbf{H}(CH_3)CO_2$, 1H), 1.49 (d, J= 7.1 Hz, $CO_2CH(C\mathbf{H}_3)CO_2H$, 3H), 1.42 (d, J= 6.9 Hz, $HOCH(C\mathbf{H}_3)CO_2$, 3H).

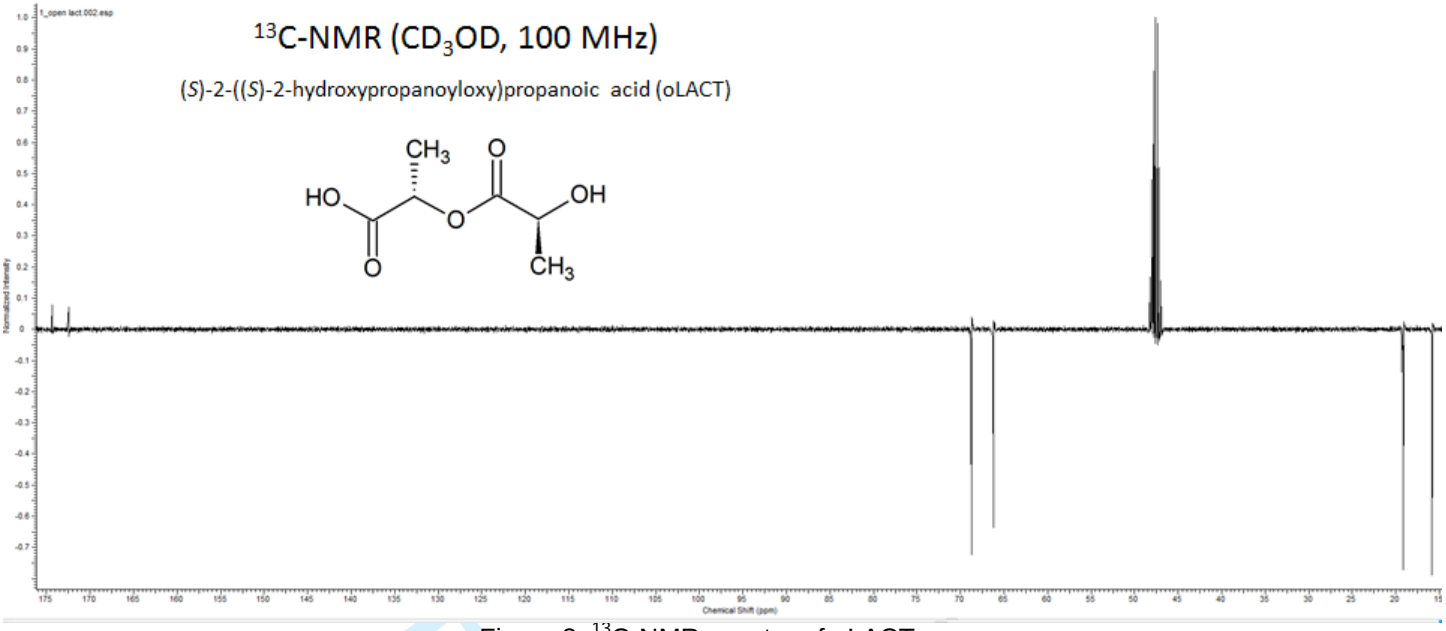

Figure 3. ^{13}C-NMR spectra of oLACT.

Spectral information:
^{13}C-NMR (MeOD-d_4): δ 174.33 (**C**O_2H, 1C), 172.44 (**C**O_2C, 1C), 68.71 (CO_2**C**H(CH_3)CO_2H, 1C), 66.21 (HO**C**H(CH_3)CO_2, 1C), 19.06 (HOCH(**C**H_3)CO_2, 1C) 15.80 (CO_2CH(**C**H_3)CO_2H, 1C).

Figure 4. HPLC-DAD analysis of the purified pLACT (λ= 260 nm).

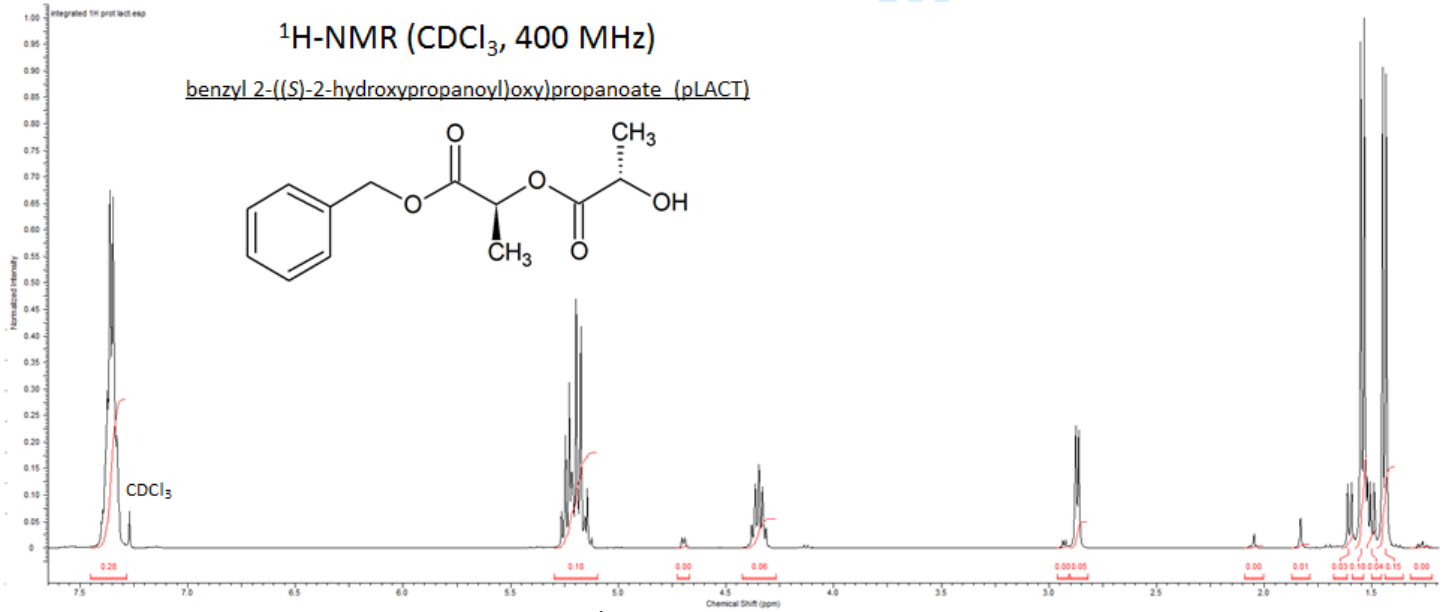

Figure 5. ^{1}H-NMR spectra of pLACT.

Spectral information:
^{1}H-NMR ($CDCl_3$): δ 7.35 (m, Ar, 5H), 5.19 (m, $CO_2C\mathbf{H}(CH_3)CO_2C\mathbf{H}_2Ph$, 3H), 4.34 (m, $HOC\mathbf{H}(CH_3)CO_2CH(CH_3)CO_2Ph$, 1H), 2.86 (d, J= 5.8 Hz, **H**O, 1H), 1.53 (d, J= 7.1 Hz, $CO_2CH(C\mathbf{H}_3)CO_2CH_2Ph$, 3H), 1.43 (d, J= 6.9 Hz, $HOCH(C\mathbf{H}_3)CO_2$, 3H).

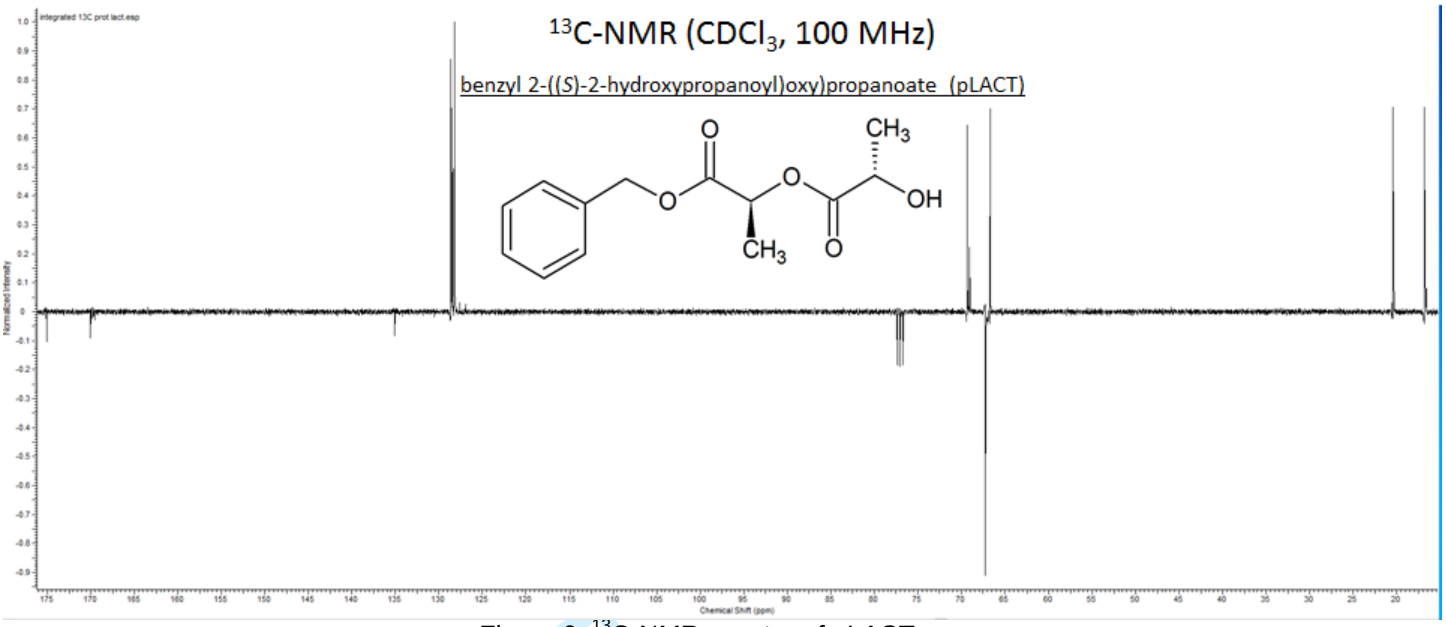

Figure 6. ^{13}C-NMR spectra of pLACT.

Spectral information:
^{13}C-NMR ($CDCl_3$): δ 175.03 ($\mathbf{C}O_2CH_2$, 1C), 170.00 ($\mathbf{C}O_2C$, 1C), 135.04 (Ar-*ipso*, 1C), 128.60 (Ar-*meta*, 2C), 128.49 (Ar-*para*, 1C), 128.20 (Ar-*ortho*, 2C), 69.36 ($CO_2\mathbf{C}H(CH_3)CO_2CH_2Ph$, 1C), 67.20 ($CO_2CH(CH_3)CO_2\mathbf{C}H_2Ph$, 1C), 66.66 ($HO\mathbf{C}H(CH_3)CO_2$, 1C), 20.36 ($HOCH(\mathbf{C}H_3)CO_2$, 1C), 16.78 ($CO_2CH(\mathbf{C}H_3)CO_2CH_2Ph$, 1C).

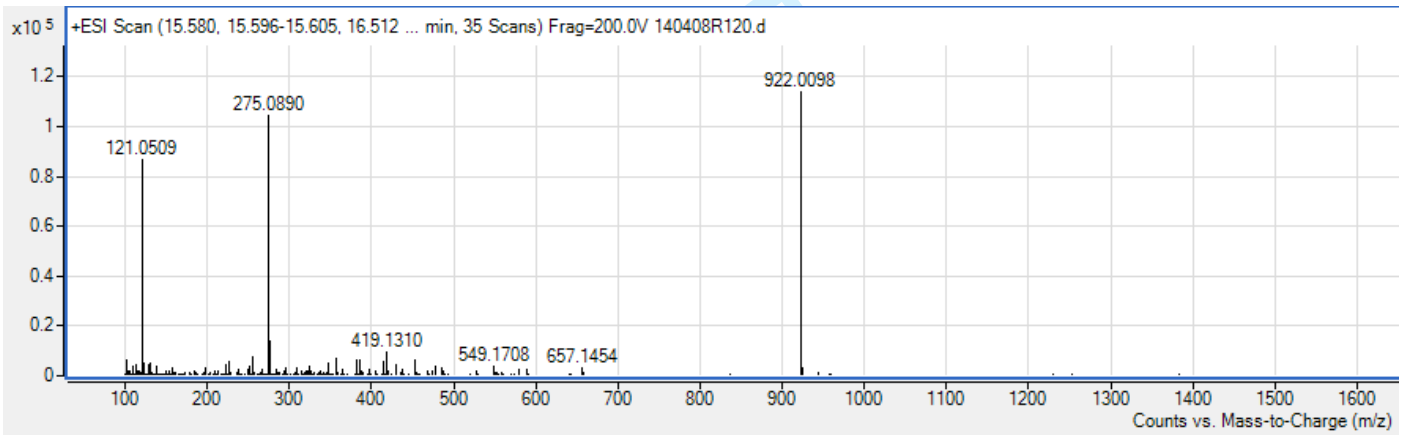

Figure 7. LC/TOF-MD analysis of the pLACT. ESI scan of the peak corresponding to the compound. $C_{13}H_{16}O_5$: calculated: $(M+Na)^+$: 275.089; found $(M+Na)^+$: 275.0890. Reference masses: 121.050873 and 922.009798 *m/z.*

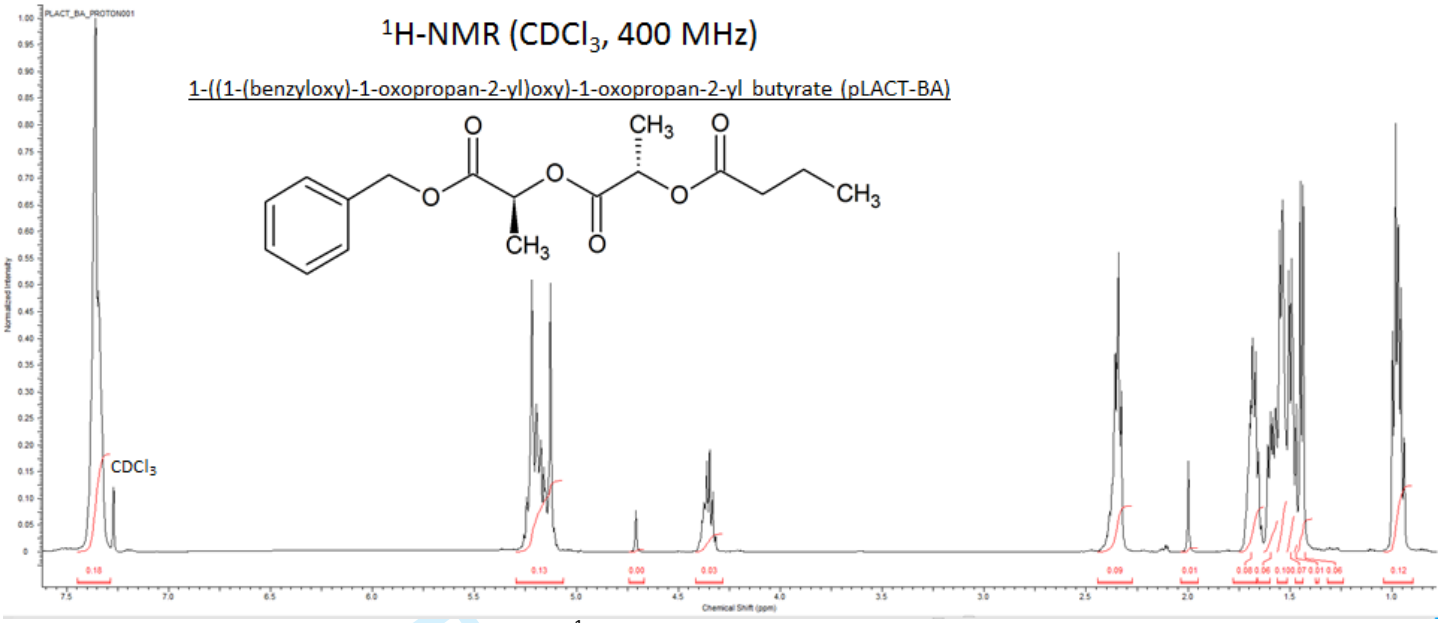

Figure 8. ^{1}H-NMR spectra of pLACT-BA.

Spectral information:

^{1}H-NMR ($CDCl_3$): δ 7.29 (m, Ar, 5H), 5.13 (m, $CO_2C\mathbf{H}(CH_3)CO_2C\mathbf{H}_2Ph$, 3H), 4.29 (q, J= 6.9 Hz, $ButyrCOOC\mathbf{H}(CH_3)CO_2CH(CH_3)CO_2Ph$, 1H), 2.25 (t, J= 7.3 Hz, $OCC\mathbf{H}_2CH_2CH_3$, 2H), 1.60 (hex, J= 7.3 Hz, $OCCH_2C\mathbf{H}_2CH_3$, 2H), 1.47 (d, J= 7.0 Hz, $CO_2CH(C\mathbf{H}_3)CO_2CH_2Ph$, 3H), 1.37 (d, J= 6.9 Hz, $ButyrCOOCH(C\mathbf{H}_3)CO_2$, 3H), 0.91 (t, J= 7.3 Hz, $OCCH_2CH_2C\mathbf{H}_3$, 3H).

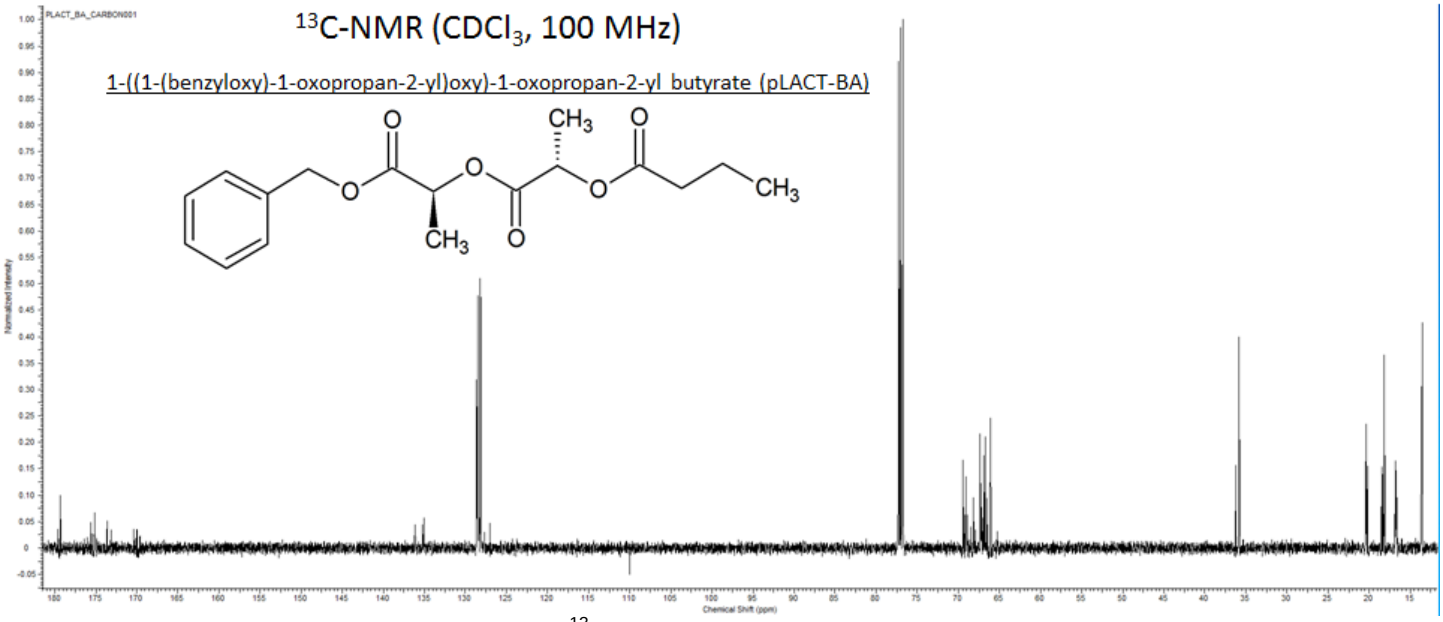

Figure 9. ^{13}C-NMR spectra of pLACT-BA.

Spectral information:

^{13}C-NMR ($CDCl_3$): δ 178.00 ($Butyr\mathbf{C}O_2C$, 1C), 174.70 ($\mathbf{C}O_2CH_2$, 1C), 170.00 ($\mathbf{C}O_2C$, 1C), 134.88 (Ar-*ipso*, 1C), 128.37 (Ar-*meta*, 2C), 128.27 (Ar-*para*, 1C), 127.95 (Ar-*ortho*, 2C), 69.02 ($CO_2\mathbf{C}H(CH_3)CO_2CH_2Ph$, 1C), 66.95 ($CO_2CH(CH_3)CO_2\mathbf{C}H_2Ph$, 1C), 66.45 ($ButyrCOO\mathbf{C}H(CH_3)CO_2$, 1C), 35.52 ($OC\mathbf{C}H_2CH_2CH_3$, 1C), 19.97 ($ButyrCOOCH(\mathbf{C}H_3)CO_2$, 1C), 17.93 ($OCCH_2\mathbf{C}H_2CH_3$, 1C), 16.53 ($CO_2CH(\mathbf{C}H_3)CO_2CH_2Ph$, 1C), 13.30 ($OCCH_2CH_2\mathbf{C}H_3$, 1C).

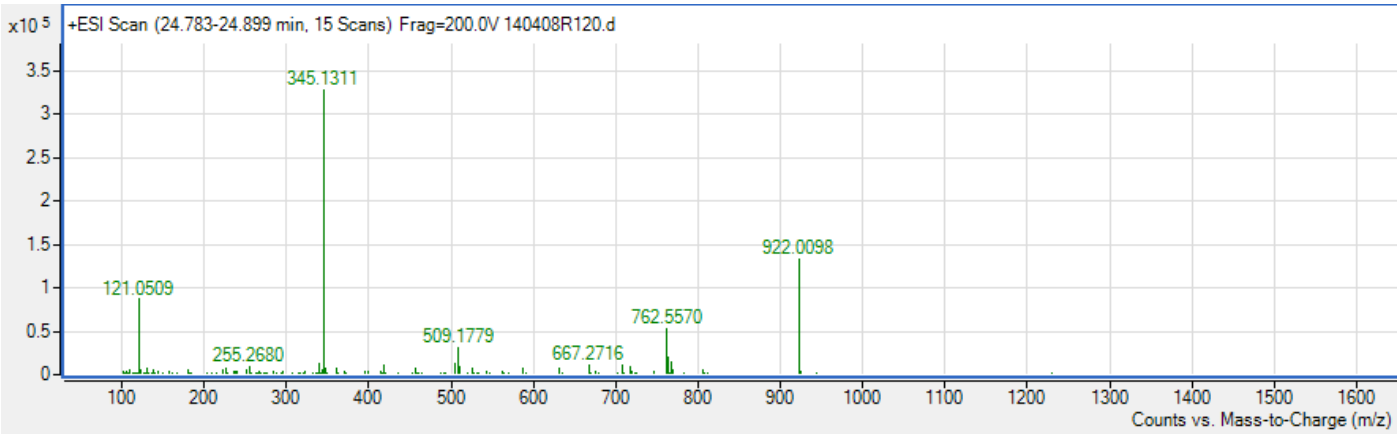

Figure 10. LC/TOF-MD analysis of the pLACT-BA. ESI scan of the peak corresponding to the compound. $C_{17}H_{22}O_6$: calculated: $(M+Na)^+$: 345.1309; found $(M+Na)^+$: 345.1311. Reference masses: 121.050873 and 922.009798 *m/z.*

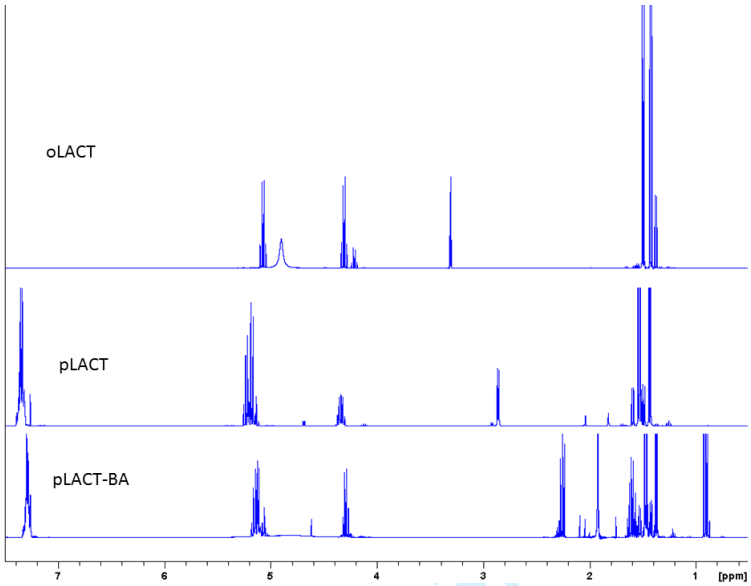

Figure 11. ^{1}H-NMR zoom for the products oLACT, pLACT and pLACT-BA.

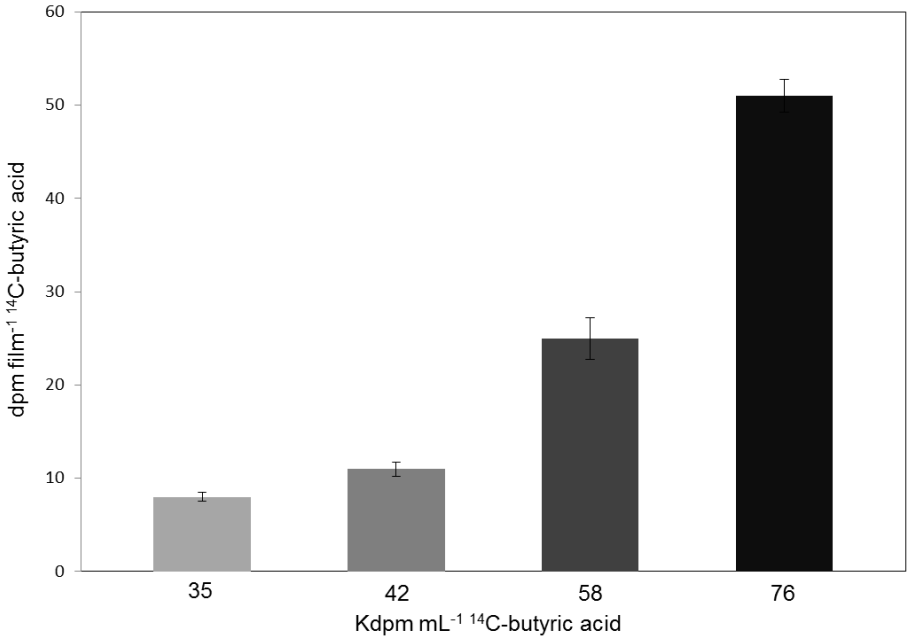

Figure 12. Sorption trend of the ^{14}C-butyric acid on the PLLA films.

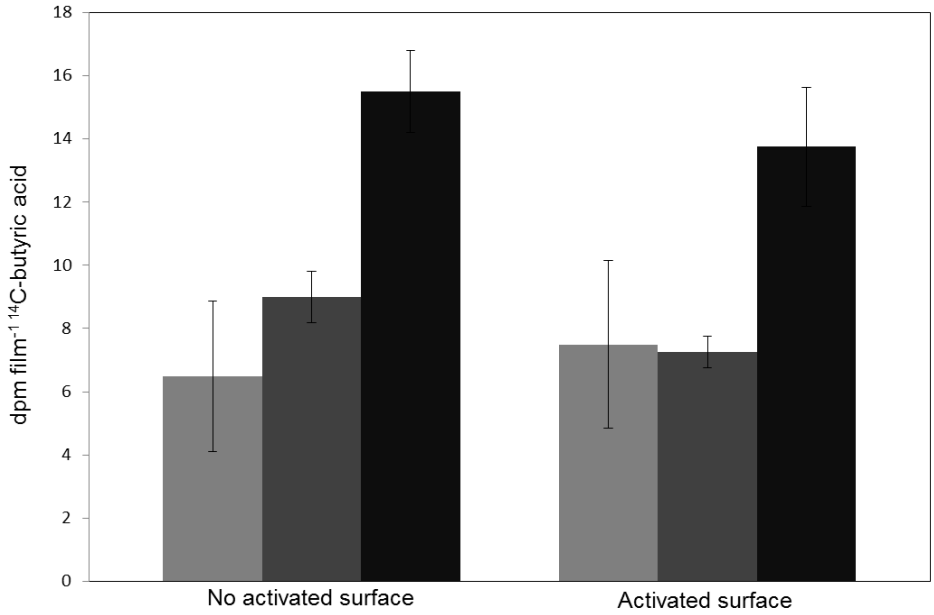

Figure 13. Effect of the enzyme on the reaction. Light grey: CTRL; dark grey: 88 U CaLB; black: 880 U CaLB.

Table S1. Roughness and waviness of the PLLA after the different activation and functionalization treatments.

Treatment		R_a (nm)[a]	W_a (nm)[a]
1st step: Activation	2nd step: Functionalization		
Starting PLLA		0.3	46.1
Buffer 24 h		0.7	61.7
Hydro 24 h		3.6	298.1
Buffer 24 h	Heptane	0.7	48.4
Hydrolyzed	Heptane	3.5	219.3
Buffer 24 h	No Enzyme	0.5	38.4
Hydrolyzed	No Enzyme	3.8	250.8
Buffer 24 h	Enzyme	0.4	48.0
Hydrolyzed	Enzyme	3.3	241.7

[a]Determined using Atomic Force Microscopy (AFM).

Table S2. Dose-effect of the enzyme (aCaLB) on the esterification of PLLA with butyric acid with and without prior enzymatic activation (i.e. limited hydrolysis). Concentration of ^{14}C-radiolabeled butyric acid of 35 kdpm mL^{-1}.

Treatment 24 h		Counts (dpm film^{-1})[a]	STD (dpm film^{-1})[a]
1st step: Activation	2nd step: Functionalization		
Buffer	No Enzyme	6.5	2.4
Hydrolysis	No Enzyme	7.5	2.6
Buffer	88 U enzyme	9.0	0.8
Hydrolysis	88 U enzyme	7.3	0.5
Buffer	880 U enzyme	15.5	1.3
Hydrolysis	880 U enzyme	13.8	1.9

[a]Determined using ^{14}C-radiochemical analysis.

Table S3. Use of different CaLB preparations in the esterification of PLLA with butyric acid with and without prior enzymatic activation (i.e. limited hydrolysis). Concentration of ^{14}C-radiolabeled butyric acid of 58 kdpm mL^{-1}.

Treatment 24 h		Counts (dpm film^{-1})[a]	STD (dpm film^{-1})[a]
1st step: Activation	2nd step: Functionalization		
X	X	25.8	2.2
hydro	X	27.5	1.0
X	aCaLB	50.8	4.6
hydro	aCaLB	46.8	3.3
X	cCaLB	51.0	1.7
hydro	cCaLB	56.0	2.9

[a]Determined using ^{14}C-radiochemical analysis.

Table S4. XPS analysis of the different enzymatic treated PLLA samples.

Sample		% C	% O	% N	% Na	% F	% Mg
Native PLLA		67.64	32.36	n.d.	n.d.	n.d.	n.d.
24 hydrolysis		62.11	37.15	n.d.	0.74	n.d.	n.d.
24 h hydrolysis	24 h CTRL	61.62	36.86	0.36	1.05	0.19	0.29
24 h hydrolysis	24 h 880 UCaLB	66.32	30.86	1.58	0.85	0.40	n.d.

List of abbreviations

AcN	Acetonitrile
AFM	Atomic Force Microscopy
ATR-FTIR	Attenuated total reflection-Fourier transform infrared spectroscopy
BA	Butyric Acid
BB	Benzyl Bromide
BHT	2,6-bis(1,1-dimethylethyl)-4-methylphenol
BSA	Bovine Serum Albumin
CaLB	*Candida antarctica* lipase B
aCaLB	adsorbed *Candida antarctica* lipase B
cCaLB	Covalently immobilized *Candida antarctica* lipase B
fCaLB	lyophilized *Candida antarctica* lipase B
CTRL	Control reaction
DSA	Drop Shape Analysis
EDC	3-(Ethyliminomethyleneamino)-*N*,*N*-dimethylpropan-1-amine
fCaLB	Lyophilized *Candida antarctica* lipase B
GPC	Gel Permeation Chromatography
HiC	*Humicola insolens* cutinase
HPLC-DAD	High Performance Liquid Chromatography-Diode Array Detector
HPLC-RI	High Performance Liquid Chromatography-Refrective Index
HSA	Human Serum Albumin
LC/ESI/TOF/MS	Liquid Chromatography/Electrospray Ionization/Time of Flight/Mass
NMR	Nuclear Magnetic Resonance
oLACT	Opened Lactide
PCL	poly(caprolactone)
PET	poly(ethylene terephthalate)
PLA	poly(lactic acid)
pLACT	Protected Lactide Dimer
pLACT-BA	Protected Lactide Dimer coupled with butyric acid
PLLA	poly(L-lactic acid)
PNPB	p-nitrophenyl butyrate
SEM	Scanning Electron Microscopy
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
T_g	Glass Transition Temperature
WCA	Water Contact Angle
XPS	X-ray Photoelectron Spectroscopy

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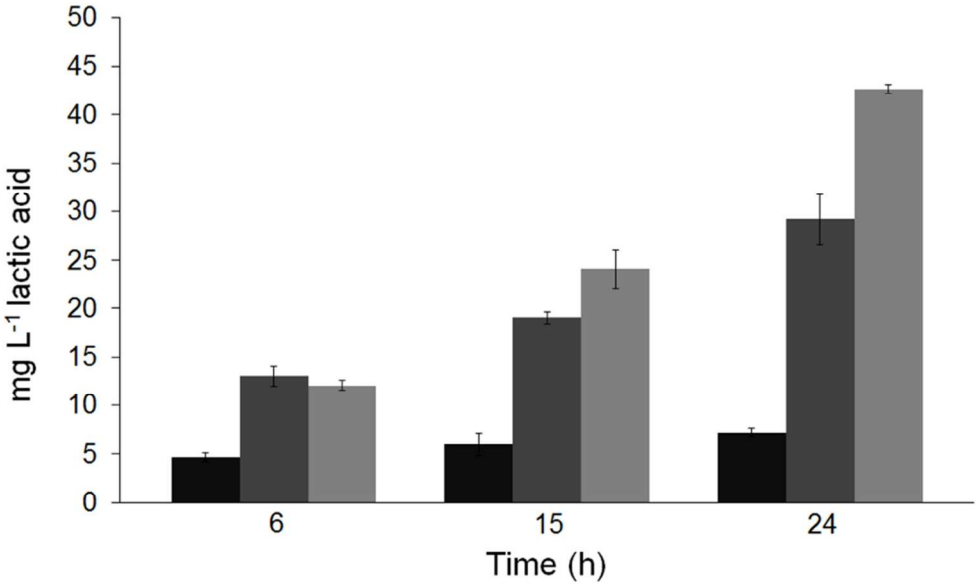

Figure 1. Lactic acid released from poly(L-lactic acid) films hydrolyzed during hydrolysis by *Humicola insolens* cutinase (HiC) in 0.1 M TRIS buffer at pH 7. Black: 1.0 mg mL^{-1} HiC; dark grey: 0.2 mg mL^{-1} HiC; gray: 0.1 mg mL^{-1} HiC. Control reactions performed using buffer solution led to no detectable hydrolysis products.
82x49mm (300 x 300 DPI)

Review

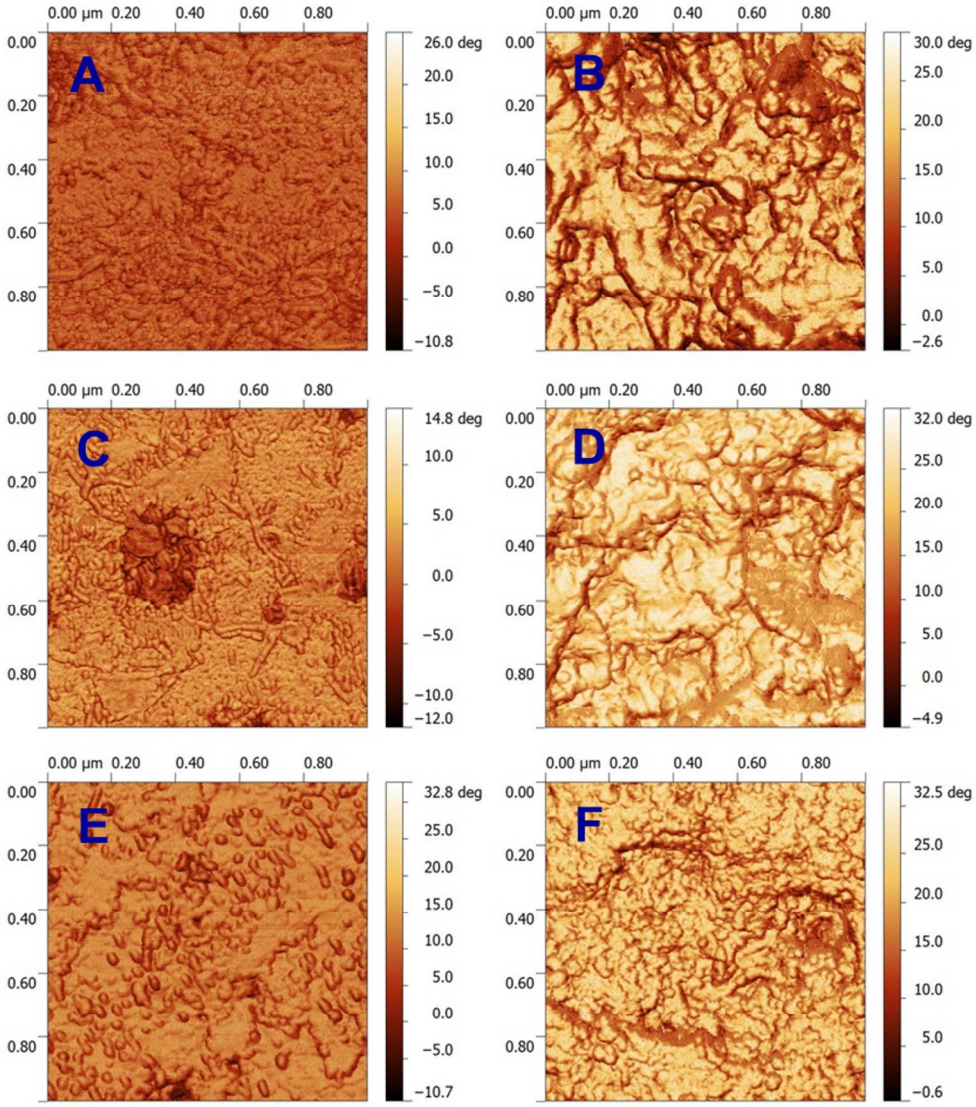

Figure 2. Atomic force microscopy phase images of the surfaces of PLLA films after enzymatic functionalization with and without enzymatic prehydrolysis. (A) PLLA blank (24 h in buffer), (B) 24 h hydrolyzed with cutinase, (C) Non-hydrolyzed/enzymatic coupling blank, (D) Hydrolyzed/enzymatic coupling blank, (E) Non-hydrolyzed/enzymatic coupling, (F) Hydrolyzed/enzymatic coupling. All AFM pictures were scanned over an area of 1 µm x 1 µm.
177x200mm (300 x 300 DPI)

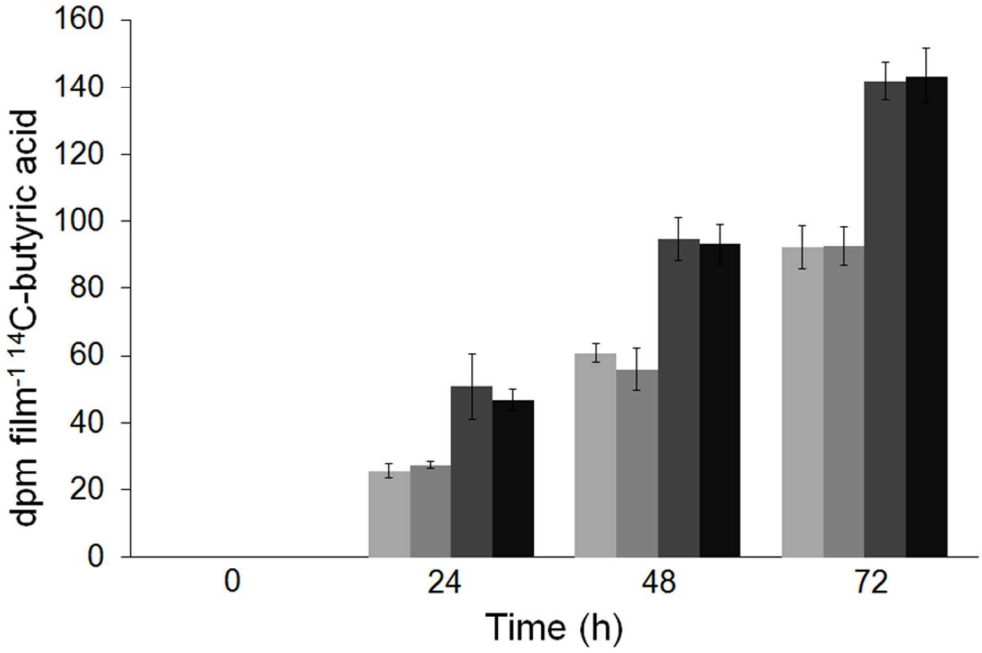

Figure 3. CaLB catalyzed functionalization of PLLA films with butyric acid with and without prior enzymatic surface hydrolysis by cutinase HiC. Light grey: CTRL-CTRL; grey: HiC-CTRL; dark grey: CTRL-CaLB; black: HiC-CaLB. The experiments were conducted using a starting concentration of ^{14}C-radiolabeled butyric acid of 58 kdpm mL^{-1}.
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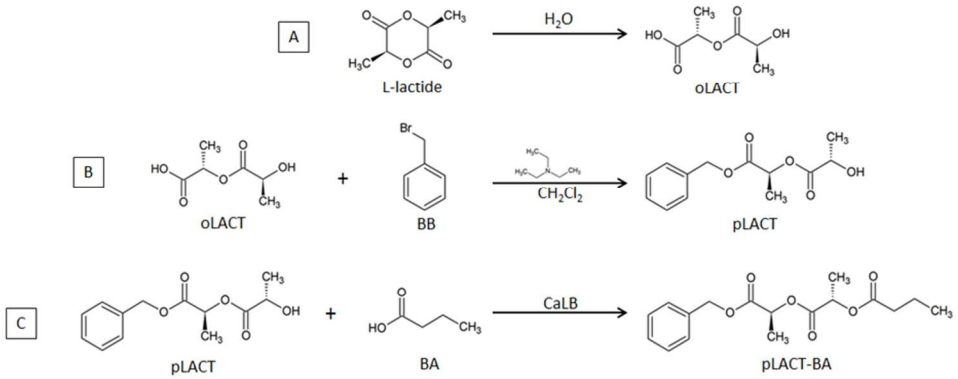

Scheme 1. Reaction scheme of the pLACT-BA synthesis. A: Ring opening of L-lactide in order to obtain oLACT. B: Protection of the -COOH end group of the oLACT using benzyl bromide (BB) in order to obtain the protected dimer with only one functional end (pLACT). C: Enzymatic esterification of pLACT with butyric acid (BA) using CaLB as biocatalyst (reaction performed both in bulk and in n-heptane as reaction solvent).
177x75mm (300 x 300 DPI)

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