Influence of Depolymerases and Lipases on the Degradation of Polyhydroxyalkanoates Determined in Langmuir Degradation Studies

Natalia A. Tarazona, Rainhard Machatschek, and Andreas Lendlein*

Microbially produced polyhydroxyalkanoates (PHAs) are polyesters that are degradable by naturally occurring enzymes. Albeit PHAs degrade slowly when implanted in animal models, their disintegration is faster compared to abiotic hydrolysis under simulated physiological environments. Ultrathin Langmuir-Blodgett (LB) films are used as models for fast in vitro degradation testing, to predict enzymatically catalyzed hydrolysis of PHAs in vivo. The activity of mammalian enzymes secreted by pancreas and liver, potentially involved in biomaterials degradation, along with microbial hydrolases is tested toward LB-films of two model PHAs, poly(3-hydroxybutyrate) (PHB) and poly[(3-R-hydroxyoctanoate)-co-(3-R-hydroxyhexanoate)] (PHOHx). A specific PHA depolymerase from Streptomyces exfoliatus, used as a positive control, is shown to hydrolyze LB-films of both polymers regardless of their side-chain-length and phase morphology. From amorphous PHB and PHOHx, ~80% is eroded in few hours, while mass loss for semicrystalline PHB is 25%. Surface potential and interfacial rheology measurements show that material dissolution is consistent with a random-chain-scission mechanism. Degradation-induced crystallization of semicrystalline PHB LB-films is also observed. Meanwhile, the surface and the mechanical properties of both LB-films remain intact throughout the experiments with lipases and other microbial hydrolases, suggesting that non-enzymatic hydrolysis could be the predominant factor for acceleration of PHAs degradation in vivo.

1. Introduction

Polyhydroxyalkanoates (PHAs) are microbially synthesized polyesters that have undergone animal testing and clinical trials for tissue response, showing biocompatibility in various host systems. Over the years, PHA based biomaterials have been fabricated from short-chain-length variants such as poly(3-R-hydroxybutyrate) (PHB), poly(4-hydroxybutyrate) (P4HB), their copolymers poly[(3-R-hydroxybutyrate)-co-(3-R-hydroxyvalerate)] (PHBV), poly[(3-R-hydroxybutyrate)-co-(4-hydroxybutyrate)] (PHB-co-P4HB), poly[(3-R-hydroxyvalerate)-co-(3-R-hydroxyhexanoate)] (PHBBHx), as well as medium-chain-length poly(3-R-hydroxyoctanoate) (PHO) and poly[(3-R-hydroxyoctanoate)-co-(3-R-hydroxyhexanoate)] (PHOHx). To date, P4HB is the only PHA used in clinically established products. It is commercially available under the trade name TephaFLEX. P4HB-based products are exemplarily monofilaments for sutures (Monomax, Aesculap AG, Tuttingen, Germany), a mesh for hernia repair (Phasix Mesh, CR Bard, Inc., Murray Hill, NJ), a scaffold for tendon repair (BioFiber, Tornier, Inc., Edina, MN), and a scaffold for plastic and reconstructive surgery (GalaFLEX, Galatea Surgical, Inc., Lexington, MA). Nevertheless, advances in the synthesis of PHAs, in the fabrication techniques for PHA-based materials, and in the post synthesis modification/functionalization of these materials for improved elasticity and biofunctionality, are paving the way for other PHAs such as PHBV in medical applications.

The degradation function of a material is a key feature for many medical applications. For this reason, studying the degradability of PHAs under conditions reflecting their designated application is compulsory to predict the material lifetime, while understanding the mechanisms leading to the loss of mechanical properties. One of PHAs major advantages comes from the ester bonds in the polymer backbone, which, upon hydrolysis generate carboxylic acids that are considerably less acidic and less inflammatory than the ones released by established degradable polymers such as polylactides. Yet, since fragmentation
proceeds via hydrolysis of ester bonds, a slow degradation of polyesters is expected under non-catalyzed conditions, with an approximate half-life of each ester bond of 3.3 years in water at pH = 7 and 25 °C.\(^\text{[20]}\) Given that PHAs are semicrystalline polymers, the crystalline regions, which are impermeable to water, further decelerate the hydrolysis reaction. Moreover, degradation-induced crystallization is expected to affect the local crystalline environment and thus the degradation rate.\(^\text{[21]}\)

Studies to determine the mechanisms governing PHAs degradation in vitro have concluded that abiotic hydrolysis proceeds with acceptable rate only under strongly acidic or alkaline conditions (pH > 12), or high temperatures.\(^\text{[22–26]}\) In long-term in vitro studies under conditions that simulate the body physiological environment (37 °C and pH = 7.4), the mass loss of PHB, PHBH, and PHO films was insignificant (between 0% and 8%) up to two years.\(^\text{[6,27–29]}\) Under the same conditions, there was a considerable decrease in the molecular weight of about 30% in 6 months,\(^\text{[28]}\) = 40% in 1 year, or = 65% in two years\(^\text{[27]}\) for high molecular weight PHB (>500 000 Da), and = 70% for PHO.\(^\text{[29]}\) Similarly, PHBH copolymer in water at 25 and 30 °C showed a molecular weight decrease of = 30% and 40% after one year.\(^\text{[25]}\) However, in order to reach the stage where substantial mass loss occurs, the molecular weight has to drop to a value close to the solubility limit, which is probably below 1 kDa, corresponding to a 99.8% reduction in molecular weight for the aforementioned PHAs.

On the other hand, long-term in vitro tissue response studies, in vivo studies on animal models and clinical trials with patients are still not conclusive on the fate of PHAs implants and the kinetics of the processes leading to the loss of mechanical properties. Some reasons for opposing results may be the variability between sample properties, the use of different animal models, and the slow degradation of PHAs. Nonetheless, in some studies a faster disintegration compared to in vitro conditions has been observed. Hence, both pure hydrolysis and enzymatically catalyzed degradation mechanisms have been proposed for PHAs in vivo. Enzymes are large molecules that are confined to the surface of the material, meaning that enzymatic catalysis has little effect on the molecular weight of the bulk material. Yet, enzymes reduce the mass and size of the device by continuously eroding material from the surface.

Early studies on PHB and PHBH copolymer non-porous disc implants in mice, showed a 15–43% reduction in molecular weight following 6 months of implantation, while no changes in the implants initial appearance and only 1.6% maximal mass loss of their initial weight were observed.\(^\text{[8]}\) Later on, computed tomography examinations of human patients after heart surgery with PHB pericardial patches, fabricated as microfilaments forming a web-like structure, showed a statistically significant decrease in the mean patch volume by 27% after 24 months.\(^\text{[30]}\) The time for complete degradation was not specified, but polymer remnants were seen after 24 months, while some macropores were present around the implant after 30 months.\(^\text{[31]}\)

The mechanical properties of non-porous PHB samples (210 000 Da), explanted after 1, 2, 4, 26, and 52 weeks of subcutaneously and intraperitoneally permanence in male Wistar rats, suggested that degradation of PHB proceeds as a surface erosion process determined by a decrease in microhardness and apparent increase of crystallinity in the first 4 weeks of implantation. The molecular weight decreased slowly to 50%, while 75% of the original bending strength (bulk property) was still retained by the implant after 52 weeks.\(^\text{[7]}\)

The molecular weight analysis of implanted PHB atactic-PHB (at–PHB) patches in rat gastrointestinal showed a fast initial molecular weight decrease followed by a deceleration after two weeks. Only one of the 4 implanted patches remained unab sorbed after 26 weeks, and the retrieved patch remnant appeared heavily decomposed with a molecular weight of about 38% of the initial value.\(^\text{[26]}\) The fast decrease in molecular weight was related to an increase of mRNA encoding pancreatic enzymes found after implantation of PHB patches onto the gastric wall of rats.\(^\text{[32]}\) The effect of pancreatic enzymes (a mixture of enzymes including lipase, amylase, α-chymotrypsin, trypsin and protease) was tested in vitro, resulting in a differential molecular weight loss of PHB films when compared to the control sample incubated on PBS. However, no mass loss was observed.\(^\text{[6]}\)

In the in vivo hydrolysis of PHB Tepha Inc. materials, implanted either subcutaneously or intramuscularly in animal models, occurs primarily by bulk hydrolysis; however, surface erosion and pitting of devices was observed and suggested being facilitated by an enzymatic component.\(^\text{[16]}\) The molecular weight of a PHASIX mesh and a P4HB plug material for soft tissue repair in porcine models decreased progressively, 33% and 83% reduction in 12 and 52 weeks, while the implants remained visible and intact at the repair sites until the end of the study (52 weeks).\(^\text{[32,33]}\) After implantation, the molecular weight of these devices decreased in an almost linear fashion, with a rate of about 2% per week. After one year, only fragments or fibers can occasionally be recovered.\(^\text{[34]}\) In a clinical trial with 150 patients, the monofilament suture material MonoMax (P4HB) was used for abdominal wall closure. The sutures retained 50% of their tensile strength after 100 days, a result of their slow absorption.\(^\text{[35]}\)

The aforementioned findings, namely a faster disintegration, but similar molecular weight reduction compared to in vitro conditions raises the question of how the degradation of PHAs is accelerated in vivo. In general, chain-scission of degradable aliphatic polyesters under physiologic conditions can be initiated i) passively by hydrolysis, ii) by enzyme-catalyzed hydrolysis or (iii) through oxidation.\(^\text{[36]}\) Although the guidelines for in vitro assessment of chemical degradation have been outlined in ASTM F1635 for surgical implants, the slow degradation of polyesters remain a major limitation.

The Langmuir-Blodgett monolayer degradation technique (LMD) allows to predict the degradation behavior and physico-chemical changes in polymers when exposed to different environments, with experimental observation time periods in the range of a few hours.\(^\text{[37,38]}\) It enables the direct observation of the particular influence of molecular (phase morphology) and external parameters (pH, enzymes, temperature) in a non-invasive manner, as a direct representation of the degradation process occurring at the surface.

Here, we hypothesize that the LMD technique allows determining whether enzymes could accelerate the degradation of β-PHAs in vivo, ruling out the course of pure hydrolysis (abiotic) outlined for two model PHAs in preceding publications.\(^\text{[25,38]}\) PHB and PHOHx films were prepared at the air-water (A-W) interface and incubated with i) a PHA depolymerase for initial modeling of enzymatic-catalyzed surface erosion, ii) a
mammalian lipase and esterase, relevant to material implant locations in vivo; and iii) commercial microbial lipases with polyester-degrading activity. The mass loss was calculated from the area reduction of the films at the A-W interface, while the molecular weight loss was followed by the surface potential and interfacial viscosity of films.

2. Results

2.1. Degradation of Amorphous PHB and PHOHHx Films by PHA Depolymerase

PHAs are degraded in nature by PHA depolymerase enzymes, secreted by many microorganisms populating the same niche as PHA producing bacteria. The extracellular depolymerase from *Streptomyces exfoliatus* PhaZ Sex2 was used for modeling of enzymatic-catalyzed surface erosion of PHAs on LMD experiments. An overview on the experimental approach followed in this study is shown in Figure 1.

The enzyme PhaZ Sex2 was found to be capable of degrading PHB and PHOHHx amorphous films, prepared on buffer pH = 10 at \( \pi_0 = 10 \text{ mN m}^{-1} \) on a medium area Langmuir trough. The surface area versus time curve in Figure 2A shows that degradation of PHOHHx is faster than that of PHB, with a calculated mass loss of 55% after 100 min, while PHB reached the same value after an incubation period of 250 min (Figure 2A). The activity of PhaZ Sex2 decreases as the medium becomes less alkaline (Figure 2A, green line). Under similar conditions, but in the absence of enzymes, we did not observe mass loss up to 20 h; but at high pH > 12, the hydrolysis of PHB with a shorter side-chain-length occurred notably faster than for PHOHHx due to steric shielding of ester bonds.[23] However, these results are not surprising given that enzymatic degradation is dependent on the affinity of the enzyme for the substrate, and PhaZ Sex2 has a strong affinity for PHOHHx.[39] Nonetheless, the considerable activity of PhaZ Sex2 toward PHB with small side-chain-length is a new insight from this study. The rate constants obtained from the fit-curves at pH = 10 in Figure 2A were \( k = 0.013 \text{ min}^{-1} \) for PHOHHx and \( k = 0.003 \text{ min}^{-1} \) for PHB, assuming the longest water-soluble fragment was a dimer in both cases. It is worth to mention that degradation could not be followed to completion due to the area required in the experimental setup for the characterization devices.

Interfacial moduli data could not be used to assess the reduction of the molecular weight of amorphous PHA films during
degradation, since no significant complex interfacial viscosity values \( \eta^* \) could be observed even after 80% mass loss (Figure S1, Supporting Information).

Nonetheless, we demonstrated in a previous paper that the surface potential (\( \Delta V \)) of (co)polyesters and its progression is highly affected by the chain fragmentation mechanism.\(^{38,40}\) To assay the effect of PhaZSex2 on the surface potential of PHB, an amorphous PHB layer was prepared at \( \pi_0 = 4.0 \text{ mN m}^{-1} \), which provides high sensibility for tracking \( \Delta V \) changes (Figure 2B). An initial surface potential of 420 mV was recorded for amorphous PHB during 10 min. After stabilization, PhaZSex2 was injected under the subphase at a final concentration of 0.2 \( \mu \text{g mL}^{-1} \), causing a relatively fast decrease in area (\( A/A_0 \)) due to efficient hydrolysis of ester bonds and production of water-soluble fragments. This mass loss was preceded by a very fast decrease of the surface potential. The conversion of ester bonds to carboxylic acid groups leads to generation of negative charges and thus a drop of the surface potential. The molecular weight is inversely proportional to the number of end-groups, meaning that the drop in surface potential is closely related to the drop in molecular weight. Mass loss sets in when the molecular weight is close to the solubility limit. Then, the molecular weight of the layer stabilizes, because further chain-cuts lead to immediate removal of molecules. The stabilization of the molecular weight in this mass loss phase is expressed by the stabilization of the surface potential. We note that such a solubility controlled degradation behavior is also expected for polysteres undergoing bulk degradation.\(^{41}\)

In contrast, during the hydrolytic degradation of PHB and PHOHhx films at \( \text{pH} > 12 \) we reported that the area decreased in parallel with the monolayer surface potential.\(^{23}\)

### 2.2. Degradation of Semicrystalline PHB Films (\( \approx 30\% \)) by PHA Depolymerase

To determine if PhaZSex2 could catalyze the degradation of semicrystalline PHB films, which is a more common phase morphology in nature and for any technological application, or whether its activity is limited to entirely amorphous materials, PHB films with about 30% crystallinity at \( \text{pH} = 7.4 \) (physiological \( \text{pH} \)) and \( \text{pH} = 10 \) (maximum enzymatic activity) were prepared (Figure 3). The crystallinity of the films is expressed as the relative percentage change in area after 5 h crystallization.

Cristallization of the films is accompanied by a strong increase in \( \eta^* \) at \( \pi_c = 12 \text{ mN m}^{-1} \) (Figure 3, grey section). After a stable \( \eta^* \) was reached, the polymer degradation was induced by injection of PhaZSex2 in the subphase (0.2 \( \mu \text{g mL}^{-1} \)). Degradation of PHB proceeded similarly at both pHs. In the first stage, the hydrolysis of ester bonds caused a small decrease of the normalized surface area of about \( \approx 5-10\% \) displaying a S-shape. In parallel, the interfacial viscosity decreased sharply (Figure 3, white section). This behavior is typical of a random bond-scission, and was considerable more pronounced at the optimal pH of PhaZSex2 (\( \text{pH} = 10 \)). The second stage reflects a decrease in surface area and a quick rise in the \( \eta^* \). While the transition is very fast at \( \text{pH} = 10 \), at \( \text{pH} = 7.4 \), there is a clear step between the two S-shaped area reduction processes.

The sharp rise of the modulus indicates that the second step is not a consequence of material dissolution, but rather a

Figure 3. Degradation of crystalline PHB by PHA depolymerase. In situ rheology of PHB at \( \pi_c = 12 \text{ mN m}^{-1} \) (30% crystalline film) and \( \text{pH} = 7.4 \) (solid lines) or \( \text{pH} = 10 \) (dashed lines) plus PhaZSex2 (0.2 \( \mu \text{g mL}^{-1} \)). Area reduction as a function of time (black), complex interfacial viscosity \( \eta^* \) as a function of time (orange). Time 0 and \( A/A_0 \) 100% corresponds to the area of the polymer at the time of the enzyme injection.
consequence of chain recrystallization following chain-scission (chemi-crystallization), similarly to the effect observed for PHB degradation at the A-W interface under highly alkaline conditions (pH = 13). The chemi-crystallization restrained further degradation by the enzyme, resulting in a maximum mass loss of 25% and 20% at pH = 7.4 and pH = 10 after 250 min. The possibility that the increase was an effect of enzyme penetration into the PHB layer was ruled out (Figure S1, Supporting Information).

2.3. Degradation of Amorphous and Semicrystalline (≈30%) PHA Films by Lipases

Commercial lipases produced by microorganisms, an esterase from porcine liver and the lipase preparation from porcine pancreas (Table S1, Supporting information) were used for LMD experiments using PBS (pH = 7.4 or pH = 9) at 21 °C as the subphase. Under the conditions used in this work, none of the lipases showed activity neither against amorphous PHOHx nor PHB, nor against 30% crystalline PHB, as we did not observe a significant decrease in area or in interfacial viscosity after incubation with the enzymes (Figures 4 and 5). Nonetheless, we noticed some intriguing characteristics from the lipase-PHAs interaction, such as differential adsorption depending on the side-chain of the polymers, and their phase morphology. See Figure S2 in the Supporting Information for additional graphs with all the enzymes used in this study. One group of enzymes, including lipase from P. cepacea (LPC) had no apparent surface activity or interfacial viscosity when tested on PBS at the concentration used for the LMD experiments (Figure 4A). However, the enzyme was able to insert rather quickly into amorphous PHB films crystallized at πC = 12 mN m⁻¹ for 4 h, brought to πD = 7.5 mN m⁻¹ and incubated with lipase (2 µg mL⁻¹). A/A₀ 100% corresponds to the area of the polymer at the time of the enzyme injection.

Figure 4. Langmuir degradation experiments of PHA with microbial lipases (Lipase from P. cepacea) Surface pressure (blue), area reduction A/A₀ (black), and complex interfacial viscosity η* (orange) as a function of time for: A) LPC (2 µg mL⁻¹) in PBS buffer (pH = 7.4, 21 °C), PHOHx amorphous layer at πD = 10 mN m⁻¹ after injection of lipase (2 µg mL⁻¹), and PHB amorphous film at πD = 7.5 mN m⁻¹ plus lipase (2 µg mL⁻¹). Degradation curves are shown from the injection point of lipase (A₀). The surface pressure was normalized to highlight the differences in the absorption patterns. B) PHB films crystallized at πC = 12 mN m⁻¹ for 4 h, brought to πD = 7.5 mN m⁻¹ and incubated with lipase (2 µg mL⁻¹). A/A₀ 100% corresponds to the area of the polymer at the time of the enzyme injection.

Figure 5. Langmuir degradation experiments of PHA with microbial lipases (Lipase from porcine pancreas) A) surface pressure π (blue) as a function of time for PHA amorphous films (πD = 6 mN m⁻¹) incubated with LPP (3.6 µg mL⁻¹) or for the enzyme absorbing to a bare A-W interface (PBS buffer, pH = 7.4, 21 °C). Complex interfacial viscosity η* evolution (orange) for PHOHx film plus LPP (3.6 µg mL⁻¹). Degradation curves are shown from the injection point of lipase (A₀). The surface pressure was normalized to highlight the differences in the absorption patterns. B) PHB films crystallized at πC = 12 mN m⁻¹ for 4 h, brought to πD = 7 mN m⁻¹ and incubated with LPP (3.6 µg mL⁻¹). A/A₀ 100% corresponds to the area of the polymer at the time of the enzyme injection.
PHB, and PHOHHx films, causing an increase in the surface pressure of 0.7 and 3 mN m\(^{-1}\) respectively (Figure 4B). No decrease in the film area due to degradation was observed, even after two days; on the contrary, the PHB film started losing stability, causing an increase in the \(\eta_s\) after 20 h, probably due to time-dependent crystallization. It is worth to mention that under the same conditions, LPC degrades poly(\(\varepsilon\)-caprolactone) within 3 hours. When the activity of LPC was tested against \(\approx 30\%\) crystalline PHB, prepared at \(\pi_c = 12\) mN m\(^{-1}\), no insertion was observed (Figure 4B). Instead, only a decrease of \(\approx 10\%\) in area was detected, which corresponds to area loss due to the ongoing crystallization process.

The second group of enzymes are those with relatively high surface activity, such as the purified preparation of lipase from porcine pancreas (LPP), the lipase from \(M.\ miehei\) and the esterase from porcine liver, which were inserting into the polymer films in the amorphous state right after injection into the subphase, preferentially into PHOHHx (Figure 5). The insertion of the lipase caused an increase in the surface pressure from 6 to 13 mN m\(^{-1}\) and from 6 to 78 mN m\(^{-1}\) for PHOHHx and PHB after 16 h. Further increase in surface pressure induced an increment in the interfacial viscosity, caused by the presence of the enzyme at the A-W interface as seen in the control of the lipase alone. This is supported by the results obtained with PHOHHx on PBS, in which no interfacial viscosity was observed with time. The adsorption of the protein to the A-W interface was substantially different when compared to the interface with preformed PHA films, as the moduli increased sharply after 250 min corresponding to a surface pressure of 1.5 mN m\(^{-1}\). These results indicate the existence of an intermediate stage where the protein and the polymer are interacting, rearranging the polymer molecules at the interface, until it collapses (>16 h) making space for the protein to adsorb to the surface. Since insertion of the protein could hinder the interpretation of the degradation results, half of the enzyme (1.8 \(\mu\)g mL\(^{-1}\)) was used for the experiments with 30% crystalline PHB. LPP was unable to insert into the PHB crystalline films allowing to maintain a stable film over the course of the experiment. However, as observed with the amorphous films, LPP was unable to degrade PHB, which showed intact rheological properties after 23 h (Figure 5B).

Given that the lipase solution was subjected to solvent extraction during purification, we used PLGA as a positive control for the activity of the enzyme. Under the same conditions, PLGA films were degraded within 1 h to a mass loss of 60% (Figure S3, Supporting Information).

3. Discussion

The degradation function of biomaterials is a key concern for their application in medicine. PHAs have been shown to undergo hydrolysis in vivo, albeit very slowly, while their degradation products are assimilated and metabolized by cells. Polymers of natural origin have the intrinsic ability to be degraded by naturally occurring enzymes, which may enhance the susceptibility of these materials to be metabolized under physiological mechanisms. Hydrolysis reactions of polymeric biomaterials may be catalyzed by cell-derived enzymes known as hydrolyases (lipases, proteases, esterases, glycosidases, and phosphatases, etc.), which participate in several reactions in the human body. These enzymes exist for instance in the plasma, the interstitium space, in the brush border membrane and lumen of the gastrointestinal tract, among others, to facilitate the absorption of nutrients and solutes.\(^{[42]}\) Lipases for instance are secreted by macrophages,\(^{[43]}\) which comprise a component of the cellular infiltrate after implantation of PHAs materials. However, enzymatic degradation cannot be easily addressed in vivo, requiring support from in vitro studies and prediction models.\(^{[42]}\) Given that PHAs are produced by microorganisms, the best knowledge on the degradation behavior of PHAs comes from studies using PHA depolymerases, which are also produced by microbes. Moreover, the catalytic domain of these PHA depolymerases, characterized by the catalytic triad S-H-D (serine, histidine, and aspartate residues), where the serine is positioned in the consensus lipase-box pentapeptide Gly-X1-Ser-X2-Gly, is also present in lipases, esterases and serine proteases.\(^{[44,45]}\)

On LMD experiments, we found that a PHA depolymerase from \(S.\ exfoliatus\) K10 with proven activity against PHA polymers with medium side-chain-length, such as PHOHHx, also showed remarkable activity against side-chain-length PHB. The enzyme catalyzed the degradation of both amorphous and semicrystalline PHB thin films at different pHs via a random-scission mechanism. It is therefore suggested that the extracellular depolymerase acts upon a broader range of substrates when compared to other PHA depolymerases, which may provide an advantage to degrade PHAs in the environment and for the use of this enzyme as an asset for biotechnological applications.

Interestingly, early studies suggested that for PHB degradation, the complete absence of crystalline regions in the sample (e.g., synthetic atactic poly[\(\varepsilon\)-3HB]) polymers) could enhance the enzymatic attachment process of specific PHB depolymerases.\(^{[46,47]}\) In a more recent study, a crystallizable form of PHB was constrained in two dimensions to obtain amorphous (40–50 nm thickness) and semicrystalline (150 nm thickness) films. The former were resistant to short-term degradation by a PHB depolymerase from \(C.\ testosteroni\) over a period of 4 h.\(^{[48]}\) Conversely, our study demonstrates that the depolymerase \(S.\ exfoliatus\) K10 does not necessarily bind to crystalline domains in PHAs.

Lipases and PHA depolymerases are highly diverse in terms of their sequences, but share an analogous \(\alpha/\beta\)-hydrolase fold and catalytic triad, which comprise serine/cysteine, histidine, and aspartate. Another difference between them is the substrate they normally catalyze (lipids vs PHA polymers).\(^{[32]}\) In the past, few lipases from eukaryotic and prokaryotic origin were shown capable of degrading PHB and PHAs consisting of an \(\omega\)-hydroxalkanoic acid such as poly(6-hydroxyhexanoate), poly(4-hydroxybutyrate) or their blends.\(^{[32,45,49-51]}\) However, only minor changes for \(\beta\)-PHAs polymers were found in most studies; for instance 7% and 3% weight loss for PHB-co-12%-HHx and PHB, respectively, after 50 days incubation in
PBS plus lipase 100 T (Novozymes),[13] which is not significant when compared to results on pure hydrolysis of PHB in PBS.[16] Based on our results, we support the idea that PHAs composed of β-hydroxylalkanoic acid repeating units are not degraded by common microbial lipases, chosen based on previous reports in the field. We also want to emphasize that the porcine pancreatic lipase was in fact a mixture of several enzymes of porcine origin, since the solvent extraction and precipitation process used to purify the crude product is not specific for a certain protein. It is therefore concluded that none of the porcine pancreatic enzymes catalyzes the hydrolysis of PHAs at a considerable rate.

Nonetheless, we observed that some enzymes affected the molecular organization of PHB and PHOHHx films, influencing the rheological properties of the polymer LB-films. It is worth to mention that experiments performed on solution-grown lamellar single crystals of PHB incubated with a hydroltyc-activity-disrupted mutant of a PHB-depolymerase, detected a distortion on the molecular packing of the PHB polymer chain around the loose chain packing region in the single crystal, resulting in fragmentation. Yet, no degradation of the polymer was possible by the mutant enzyme.[52]

On the other hand, inflammatory cells such as leukocytes and macrophages are also able to produce highly reactive oxygen species (ROS), such as superoxide (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), nitric oxide (NO), etc. The effect of these species on the degradation of absorbable sutures made of synthetic aliphatic polyesters has been demonstrated before.[42,51,54] Hence, it is possible that oxidative damage by ROS is the main cause of accelerated degradation of PHAs in vivo.

4. Conclusion

The PHA depolymerase from Streptomyces exfoliatus degraded PHA films regardless of their side-chain-length and phase morphology (amorphous and semicrystalline). While >80% of the polymer eroded from amorphous PHB and PHOHHx films, the maximum mass loss for semicrystalline PHB was reduced to 25%. The enzymatic degradation followed a random-scission mechanism, where a decrease in the molecular weight of the films preceded the mass loss. The surface and the mechanical properties of both PHA polymers assayed in this work remained intact after incubation with mammalian lipases, opposite to PLGA that was degraded by hydrolases from porcine pancreas. Our results show the importance of using modern interfacial studies to understand the governing mechanisms behind polymer degradation, and ratifies that non-enzymatic hydrolysis is the main mechanisms of β-PHAs in vivo disintegration. Since PHAs degradation products are not toxic and are most likely phagocytosed by macrophages in vivo, these findings support the use of pure β-PHAs polymers for long-term applications, where full degradation is not the primary expected outcome during the first years of use. If fast hydrolysis of PHAs is required in certain applications, this drawback could be overcome by copolymerization, blending and filling techniques broadly reported in the literature. Furthermore, complementary studies to evaluate the role of reactive oxygen species on the acceleration of PHAs degradation in vivo could offer substantial information on the long-term degradation behavior of PHAs. In view of the demand for sophisticated and sustainable biomaterials, which partially can replace oil-based polymers, efforts should continue toward enhancing PHAs mechanical performance and degradation behavior to enable a broad range of applications—from packaging, agriculture, textiles and medicine.

5. Experimental Section

Biopolymer Synthesis and Characterization: PHOHHx was synthesized by bacterial fermentation at shaken flask level using Pseudomonas putida KT2440 and 15 × 10\(^{-3}\) M of octanoic acid as carbon source. PHOHHx production medium, culture conditions, polymer purification and characterization have been described in previous publications.[23,55,56] PHOHHx was composed of 94% 3-R-hydroxyoctanoate – 3H0, and 6% 3-R-hydroxyhexanoate – 3HHx (molar ratio), as determined by methanolysis and HPLC. The copolymer showed a melting peak centered at 58 °C upon first heating, but did not crystallize when cooled from 80 to −70 °C in Differential Scanning Calorimetry (DSC) at 10 °C min\(^{-1}\) (\(T_m = −35\) °C). 

PHB was obtained from GoodFellow (#BU391150). The polymer molecular weight (\(M_w\)) was determined by GPC as \(M_w = 160 000\) g mol\(^{-1}\) ± 10% with a PDI of 3.6 ± 0.1. The glass transition temperature (\(T_g\)), the crystallization temperature (\(T_c\)) and the melting temperature (\(T_m\)) of PHB, measured by DSC, was 5 ± 1, 48 ± 2, and 180 ± 10 °C, respectively.[24]

Poly(-rac-lactide)-co-glycolide (PLGA, Resomer RG 504 H) was purchased from Sigma-Aldrich and used as a positive control for the degradation using lipase from porcine pancreas.[49] The polymer was synthesized to obtain one chain-end terminated by an acid group, and it has a ratio lactide:glycolide of 1:1, \(M_w = 23000 ± 10\) g mol\(^{-1}\) and a \(M_n\) of 100000 ± 10% g mol\(^{-1}\).

Source of PHA Depolymerase and Lipases: PHA extracellular depolymerase from Streptomyces exfoliatus K10 DSMZ 41 693 (PhaZ Sex2) was kindly provided by Prof. Dr. Auxiliadora Prieto. Enzyme expression was achieved in Rhodococcus sp T104 (pENV0), grown in 2 × YT (yeast extract/bactotriptone/NaCl) medium supplemented with glucose (5 g L\(^{-1}\)), at 30 °C for 72 h. Recombinant PhaZ Sex2 was purified by using affinity chromatography using a Hitrap Butyl HP sepharose column (GE Healthcare).[59] Lipases from Pseudomonas cepacia (LPC), Rhizopus oryzae (LRO), Mucor miehei (LMM), Candida antarctica (LCA), protease K from (Trichirachium album), esterase from porcine liver and lipase from porcine pancreas (containing other hydrolases, such as esterases, amylases and proteases) were purchased from Sigma-Aldrich (Darmstadt, Germany). The enzymatic activities and other technical information provided by the manufacturer are listed in Table S1 in the Supporting Information. Phosphate buffered saline (PBS, pH = 7.4) was used to dissolve the lyophilized lipases. The preparation of lipase from porcine pancreas was purified from the commercial crude product. The latter was the powder remaining after extraction of homogenized tissue with cold acetone (“acetone powder”). Lipids and other impurities were removed using a solvent extraction method.[57]

Langmuir Monolayer Degradation (LMD) Experiments: Degradation experiments were performed using a small area Langmuir trough (surface area of A = 280 cm\(^2\)), or a high-compression Langmuir trough (surface area of A = 550 cm\(^2\)) with a subphase capacity of 180 and 450 mL, respectively, equipped with a water level compensation system (Bilin Scientific, Espoo, Finland). For surface potential measurements a Kibron trough was used, consisting of a vibrating plate potentiometer coupled to a MicroTrough G2, with available surface area of 280 cm\(^2\), and a subphase capacity of 200 mL (Kibron, Helsinki, Finland). All rheology experiments were performed with the high-compression trough. The changes in the surface tension at the air–water A-W interface (surface pressure) after spreading the (co)polymers from a chloroform solution and during degradation were monitored by a Wilhelmy plate.
microbalance. The (co)polymers were prepared on a PBS subphase at a final concentration of $C_f = 0.04 \mu g \text{ mL}^{-1}$, and later exposed with a constant compression rate of 10 mm min$^{-1}$ until the optimal surface pressure for degradation experiments $\pi_{exp}$ was reached. For amorphous films, $\pi_{exp}$ was brought around the maximum compressibility modulus of the layer, which for PHB Langmuir films falls between 6 and 10 mN m$^{-1}$, while for PHOHx was found between 6 and 12 mN m$^{-1}$ at pH < 12. To prepare crystalline PHB film, $\pi_{exp}$ was brought to the PHB crystallization surface pressure $\pi_c = 12 \text{ mN m}^{-1}$, which was established as an increase in complex interfacial viscosity $\eta^c$. After crystallization, the film was allowed to stabilize around 5 h before degradation was induced. During this time, the area reduction due to crystallization reached ~30% PHOHx does not crystallize at the A-W interface, instead a 2D to 3D transition at 16 mN m$^{-1}$ was observed.

LMD experiments were performed under isobaric conditions to maintain a constant areal concentration of repeat units. To induce degradation, enzymes were injected under the films at concentrations between 0.2 and 4 $\mu g$ mL$^{-1}$ (Table S1, Supporting Information). The activity of PhaZ$_{sex2}$ depolymerase was tested on films preformed on PBS at pH = 8 or pH = 10, optimal for the enzyme activity. Similarly, the activity of the lipase from M. miehei was assayed at pH = 9. For the rest of the lipases, PBS pH = 7.4 was used. The temperature of the subphase was kept constant at 21 ± 1°C.

Rheology Experiments: Rheology experiments were carried out with an Interfacial Shear Rheometer (IRS, model MCR502) from Anton Paar (Austria), which consists of a biconical disk with a radius of $r = 25.5 \text{ mm}$ coupled to a driving motor, and to a torque and normal force transducer unit. The edge of the bicone was placed in the interface of the high-compression trough. Measurements were carried out at defined strain of 8% (assuming an edge-wall distance of 3.5 mm when a circular cap was used) and an oscillation frequency of $\omega = 0.1 \text{ rad s}^{-1}$. An algorithm implemented in the Rheocompass software was used to convert the measured bulk properties into interfacial values. For degradation experiments, the distance between bicone and trough edge (without circular cap) was set to 100 mm when calculating the interfacial moduli using the aforementioned algorithm. The storage modulus ($G'$) accounts for the elastic component, and the loss modulus ($G''$) for the viscous component of the response to oscillatory shear. The analysis of the crystallization and degradation behavior was based on the complex interfacial viscosity $[\eta^c]$ as the relevant parameter describing the viscoelastic response, with $\eta^c = [\eta^c] - [\eta^c - \rho^c_{exp}] G_c G^*$.

Surface Potential versus Mean Molecular Area Isotherms: For surface potential $\Delta \psi$ measurements, the vibrating plate was placed $2 \text{ mm}$ above the water surface in the Kibron trough. The surface potentiometer’s value was set to zero before deposition of the monolayer on the surface. The polymer was from a chloroform solution reaching a final concentration in the trough of $G_f = 0.05 \mu g \text{ mL}^{-1}$. Before compression of the films, the surface pressure and the surface potential were allowed to come to equilibrium for $\approx 10 \text{ min}$. The Langmuir layers were symmetrically compressed at constant compression rate of 10 mm min$^{-1}$. The enzyme was injected under the barriers at a final concentration in the trough of 0.4 $\mu g$ mL$^{-1}$ since it generates concentration gradients.

Error Considerations: All presented data correspond to individual experiments. Degradation curves were generally very reproducible, and the representation as normalized data removes any fluctuations arising from differences in concentrations or volumes. The random errors of an area reduction curve as the outcome of a degradation experiment were minimal. The error was determined by fluctuations in surface pressure and the precision of area measurement

$$\Delta A_{rough} = \left[ \frac{dA}{dA_{exp}} \right]^{-1} + \Delta \pi + \Delta A_{rough}$$

In the following, the calculation of the error was exemplified for the high compression trough: For PHOHx at 8 nN m$^{-1}$, it was found that:

$$\left[ \frac{dA}{dA_{exp}} \right] = \frac{0.13 \text{nN m}^{-1}}{\text{cm}^2}$$

The fluctuations of the surface pressure during the degradation experiments were $\Delta \pi = 0.005 \text{nN m}^{-1}$. The precision of the area recording of the high compression trough was $\Delta A_{rough} = 0.001 \text{cm}^2$.

Then, $\Delta A_{rough} = -0.05 \text{ cm}^2$. Typically, during degradation experiments, the area reduced from about 400–100 cm$^2$, meaning the relative error was between 0.05% and 0.01%. In contrast to the quantitative measurements of the mass loss of the area, surface potential and rheology measurements are only of relative meaning. The absolute value of the surface potential is strongly dependent on the starting configuration, e.g. the distance between probe and film. The rheometer requires a circular slit around the bicone to allow for deducing absolute interfacial moduli, but this geometry is not suitable for enzymatic degradation experiments since it generates concentration gradients.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was financially supported by the Helmholtz Association through programme-oriented funding. The authors especially thank Ing. Manuela Keller for technical support. The authors thank Prof. M. Auxiliadora Prieto and Francisco Blanco for providing the PHA-depolymerase enzyme.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

enzymatic-degradation, Langmuir thin-films, lipases, PHA-depolymerases, polyhydroxyalkanoates (PHA)

Received: May 17, 2020
Revised: June 10, 2020
Published online: July 23, 2020

[1] S. P. Valappil, S. K. Misra, A. R. Boccaccini, I. Roy, Expert Rev. Med. Devices 2006, 3, 853.
[2] Q. Wu, Y. Wang, G.-Q. Chen, Artif. Cells, Blood Substitutes, Biotechnol. 2009, 37, 1.
[3] S. F. Williams, D. P. Martin, A. C. Moses, Aesthetic Surg. J. 2016, 36, 533.
[4] M. Albertsmeier, C. M. Seiler, L. Fischer, P. Baumann, J. Husing, C. Seidlmayer, A. Franck, K. W. Jauch, H. P. Knaebel, M. W. Buchler, Langerbeck’s Arch. Surg. 2012, 397, 363.
[5] J. S. Roth, G. J. Anthone, D. J. Selzer, B. K. Poulouse, J. G. Bittner, W. W. Hope, R. G. Martindale, R. M. Dunn, M. I. Goldblatt, D. B. Earle, presented at SAGES Emerging Technology Conf., Las Vegas, April 2015.
[6] T. Freier, C. Kunze, C. Nischan, S. Kramer, K. Sternberg, M. Saß, U. T. Hopp, K.-P. Schmitz, Biomaterials 2002, 23, 2649.
[7] D. Behrend, K.-P. Schmitz, A. Haubold, Adv. Eng. Mater. 2000, 2, 123.
[8] S. Gogolewski, M. Jovanovic, S. Perren, J. Dillon, M. Hughes, J. Biomed. Mater. Res. 1993, 27, 1135.
[9] P. Schaefermeier, D. Szymanski, F. Weiss, P. Fu, T. Lueth, C. Schmitz, B. Meiser, B. Reichart, R. Sodian, Eur. Surgical Res. 2009, 42, 49.
