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Relation between Surface Area and Surface Potential Change during (co)Polyesters Degradation as Langmuir Monolayer

Natalia A. Tarazona[1], Rainhard Machatschek[1], and Andreas Lendlein[1,2,*]

[1]*Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstraße 55, 14513 Teltow, Germany*
[2]*Institute of Chemistry, University of Potsdam, Karl-Liebknecht-Straße 24-25, 14469 Potsdam, Germany*
**Correspondence to: Andreas Lendlein*
E-mail: andreas.lendlein@hzg.de

ABSTRACT

Polyhydroxyalkanoates (PHAs) are degradable (co)polyesters synthesized by microorganisms with a variety of side-chains and co-monomer ratios. PHAs can be efficiently hydrolyzed under alkaline conditions and by PHA depolymerase enzymes, altering their physicochemical properties. Using 2D Langmuir monolayers as model system to study the degradation behavior of macromolecules, we aim to describe the the interdependency between the degradation of two PHAs and the surface potential, which influences material-proteins interaction and cell response. We hypothesize that the mechanism of hydrolysis of the labile ester bonds in (co)polyesters defines the evolution of the surface potential, owing to the rate of accumulation of charged insoluble degradation products. The alkaline hydrolysis and the enzymatically catalyzed hydrolysis of PHAs were previously defined as chain-end scission and random-scission mechanisms, respectively. In this study, these two distinct scenarios are used to validate our model. The surface potential change during the chain-end scission of poly(3-R-hydroxybutyrate) (PHB) under alkaline conditions was compared to that of the enzymatically catalyzed hydrolysis (random-scission) of poly[(3-R-hydroxyoctanoate)-co-*(3-R-hydroxyhexanoate)] (PHOHHx), using the Langmuir monolayer technique. In the random-scission mechanism the dissolution of degradation products, measured as a decrease in the area per molecule, was preceded by a substantial change of the surface potential, provoked by the negative charge of the broken ester bonds accumulated in the air-water interface. In contrast, when chains degraded via the chain-ends, the surface potential changed in line with the dissolution of the material, presenting a kinetic dependent on the surface area of the monolayers. These results provide a basis for understanding PHAs degradation mechanism. Future research on (co)polymers with different main-chain lengths might extend the elucidation of the surface potential development of (co)polyesters as Langmuir monolayer.*

INTRODUCTION

Surface properties of biomaterials such as chemistry, topography, charge, and surface energy, influence protein adsorption and cell response, affecting the dynamic environment–material interaction. The surface potential, defined by the surface charge density and the orientation of the dipole moments, affects the bio-response and ultimately the performance of materials in tissue contact [1]. In general, a positively charged surface

increases the tissue regeneration response to the biomaterial (cell attachment and proliferation), since cell membranes are negatively charged. Varieties of surface modifications and coatings have been proposed to control the surface potential of biomaterials, tailoring the material bioinstructivity and application [2].

Understanding the surface potential of degrading polymer materials is a great challenge, considering the diversity of factors influencing this process, such as their sequence structure, their phase morphology, the preparation method, the sample geometry, and the degradation conditions (pH, temperature, enzymes). One approach is the measurement of the zeta potential of polymer dispersions, which, however, cannot be directly correlated to the progress of the degradation reaction [3].

Degradation of an implant starts at its surface. The Langmuir monolayer technique has been proven suitable to establish models for characterizing polymer degradation kinetics, as well as the evolution of the surface potential of polymers such as poly(ε-caprolactone) (PCL), and poly[(*rac*-lactide)-*co*-(glycolide)] (PLGA) during degradation [4, 5]. By measuring surface potential and the progress of the degradation reaction simultaneously *in situ*, information about the molecular degradation mechanism of polyesters and proteins have been obtained [6, 7].

With the Langmuir monolayer approach, molecularly thin films are formed at the air-water interface (A-W interface), with precise control of the area per molecule and other experimental conditions [8]. A monolayer film at the A-W interface can be treated as an assembly of molecular dipoles that can shift the potential across an interface. The surface potential, ΔV, can be correlated to the normal component of the surface molecular dipole moment, μ_z, using the Helmholtz equation: $\Delta V = \frac{\mu_z \Gamma}{\varepsilon}$ where ε is the permittivity of the medium and Γ is the areal concentration of dipoles at the interface.

In Langmuir Monolayer Degradation experiments (LMD) with polymers, ultrathin films of macromolecules are hydrolyzed forming water-soluble degradation products, causing a decrease in the areal concentration of repeat units. This causes a decline of the surface pressure, which is measured by a surface tension sensor coupled to the Langmuir trough [9]. Simultaneously, when (co)polyester monolayers degrade, their surface potential is expected to change due to the generation of carboxyl groups, which can carry a negative charge and influence the surface potential. For instance, Ivanova *et al*. used a so-called 'zero-order' Langmuir trough to investigate the enzymatic monolayer degradation kinetics and the generation of charged species of di-block copolymers of poly(lactic acid) and poly(ethylene glycol). The authors reported that the dipole moment of this copolymer decreased as the enzymatic degradation proceeded [4].

Here, we hypothesize that the evolution of the surface potential of (co)polyesters is highly affected by the chain fragmentation mechanism. In a random degradation mechanism, more insoluble degradation products are accumulated at the interface in the initial reaction times, when compared to a chain-end-scission [8]. To elucidate the interdependence between degradation mechanism and surface potential, we use Langmuir monolayers of polyhydroxyalkanoates (PHAs), a class of polyesters synthesized by microorganisms. PHAs stand out due to a series of features, such as cell- and tissue-compatibility, piezoelectricity, degradability and nontoxicity of the degradation products [10]. These polyesters have been widely studied for potential *in vivo* application as implantable devices and polymeric nanocarriers for delivery of non-water-soluble drugs [11].

We have previously deduced two scenarios for the degradation of PHA (co)polymers [12], which will serve as models to resolve our hypothesis. The first scenario is based on the hydrolytic degradation of PHAs under alkaline conditions, which has been reported to follow an end-cut mechanism, dependent on the pH of the medium. By this mechanism, an initial period of fast dissolution of small degradation products is realized, which is negatively influenced by the length of side-chain of the (co)polymer. Longer side-

chains in the chain-end of PHAs create steric hindrance for the ester labile bonds attack and decrease the reaction rate. Hence, for the chain-end mechanism, we used poly(3-*R*-hydroxybutyrate) (PHB) monolayers as case-study. PHB is the most studied homopolyester from the PHA family, composed of 3-*R*-hydroxybutyrate repeating units with a short side-chain (methyl group). As a semi-crystalline polymer, its crystalline fraction melts in the range of 180 ± 10 ºC [13]. Nonetheless, PHB degradation was previously achieved under accelerated conditions using an alkaline medium [14, 15].

The second scenario is established based on the enzymatically catalyzed degradation of PHAs by specific PHA depolymerases. PhaZ$_{Sex2}$ is an extracellular 27.6 kDa depolymerase secreted by *Streptomyces exfoliatus* K10 DSMZ 41693, able to degrade PHAs with high specificity, more likely by a random-scission mechanism [16]. PhaZ$_{Sex2}$ has high activity for poly[(3-*R*-hydroxyoctanoate)-*co*-(3-*R*-hydroxyhexanoate)] (PHOHHx), a copolymer with pentyl and propyl groups as side-chain. In this mechanism, the generation of water-soluble fragments takes longer time, when compared to the chain-end-scission, resulting in an initial period of slow dissolution.

Here, we first evaluated the surface potential ΔV of PHB at different pH by means of Langmuir monolayer isotherms ($\Delta V - \text{surface pressure}$, and $\Delta V - \text{area isotherms}$), to optimize the molecular packing for the surface potential measurements during degradation. Then, we followed the evolution of the surface potential $\Delta V(t)$ during the hydrolytic degradation of PHB monolayers under alkaline conditions (pH = 12.3). Furthermore, we compared the response of the surface potential at pH = 12.3 with the enzymatically mediated degradation of PHOHHx. Finally, we applied a theoretical model, based on the Helmholtz equation, to analyze the degradation mechanism by comparing the curves of $\Delta V(t)$ and surface area, $A(t)$, obtained from the experimental hydrolysis of PHB and PHOHHx.

EXPERIMENTAL DETAILS

Biopolymer synthesis and characterization

PHB was obtained from GoodFellow (#BU391150). PHOHHx copolymer (repeating unit molar ratio 94% 3HO and 6% 3HHx), and the PHA extracellular depolymerase enzyme from *Streptomyces exfoliatus* K10 DSMZ 41693 (PhaZ$_{Sex2}$) were kindly provided by Prof. Dr. Auxiliadora Prieto. The synthesis of PHOHHx was achieved by bacterial fermentation using *Pseudomonas putida* KT2440 and 15 mM of octanoic acid as carbon source as detailed previously [17]. The heterologous expression and purification of PhaZ$_{Sex2}$ has been published before [16].

Surface potential vs. Mean Molecular Area isotherms

The surface potential ΔV, of PHA Langmuir films was measured with a MicroSpot Surface Potentiometer (Kibron, Helsinki, Finland), consisting of a vibrating plate potentiometer coupled to a MicroTrough G2 from the same company, with dimensions 80 x 405 x 5 mm (W x L x D), available surface area of 280 cm^2, and a subphase capacity of 200 mL. Compression isotherms of PHA monolayers were tested in subphases consisting of i) phosphate-buffered saline, comprising 1 mM phosphate buffer and 154 mM sodium chloride (PBS, pH = 7.4); and ii) KOH solutions prepared in Milli-Q® water (pH = 10 - 13). The vibrating plate was placed approximately 2 mm above the water surface. An internal height compensation of the surface potentiometer was performed against the bare surface of every subphase before spreading the polymer solution. The surface potentiometer's value was set to zero before deposition of the

monolayer on the surface. The polymer was spread drop-wise from a chloroform solution (0.2 mg/mL) using a microsyringe (Hamilton Co., Reno, NV, USA). The spreading volume was 50 µL, reaching a final concentration in the trough of C_f = 0.05 µg/ml. The changes in the surface tension of the A-W interface (surface pressure), were monitored by a Wilhelmy plate microbalance. Before compression of the films, the surface pressure and the surface potential were allowed to come to equilibrium for approximately 5 min. The Mean Molecular Area (MMA) for PHB and PHOHHx was calculated based on the average weight and the molar fraction of the repeating units and the surface area of the trough during compression. The Langmuir layers were symmetrically compressed with two movable barriers at a constant compression rate of 10 mm/min. The temperature of the subphase was kept constant at 22 ± 0.5 °C. The values presented correspond to individual experiment data reproducible with a random measurement error of ≈ 5% concerning the surface pressure or the MMA values for two independently repeated experiments.

Degradation experiments on the Langmuir trough

The decrease in surface area, A(t), and the evolution of the surface potential, ΔV(t), as a function of time were recorded under isobaric conditions using the MicroSpot Surface Potentiometer coupled to the MicroTrough G2 from Kibron (Helsinki, Finland). Therefore, the surface pressure, and hence the areal concentration of chain segments, is kept constant by compressing the film to compensate the decrease in the areal concentration of repeat units. Once the degradation surface pressure is reached, the corresponding area is denoted as A_0. Then, a KOH or enzyme solution is injected under the monolayer and the change in the area available per molecule is recorded simultaneously with the surface potential variation. The same volume was withdrawn from the subphase to avoid technical artefacts in the surface pressure or the surface potential measurements. Two controls were performed with the KOH and enzyme subphase in the absence of a polymer layer to identify the effect of subphase evaporation, enzyme adsorption and ionic strength on the surface potential.

Theoretical Considerations

The starting point for the analysis of the surface potential of Langmuir monolayers is the Helmholtz Equation:

$$\Delta V = \frac{\mu_z \Gamma}{\varepsilon} \quad (1)$$

Here, ε is the permittivity of the medium, $\Gamma = \frac{N}{A}$ is the areal concentration of dipoles at the interface and μ_z the component of the molecular dipole moment perpendicular to the water surface. For insoluble Langmuir monolayers, where the number of molecules does not change during compression, it is common to express the surface pressure as a function of the Mean Molecular Area $MMA = \frac{A}{N}$. Then, the surface potential is related to the MMA via:

$$\Delta V = \frac{\mu_z}{\varepsilon} * \frac{1}{MMA} \quad (2)$$

Accordingly, one should observe a linear relationship when plotting ΔV over the inverse of MMA, as long as the orientation of the molecules remains constant. Then, the slope of the plot is: $\frac{\mu_z}{\varepsilon}$.The influence of degradation on the surface potential of Langmuir films of macromolecules arises from the conversion of bonds in the chain backbone (B) into pairs of chain-ends. By bond scission, the number of repeat units N_B with $\mu_z = \mu_B$ decreases while the number of pairs of chain-ends $N_{E,E}$, increases. Because chain-ends are

always generated and dissolved as pairs, a pair of chain-ends is treated as a single unit with a dipole moment of $\mu_{E,E'}$. In the case of polyesters, it is expected that the cleavage results in a carboxyl and a hydroxyl group. The former can have a negative charge, depending on the pH of the surrounding medium. Here, we will not discuss the details of surface charge and dipole moments of charged chain-ends, and just note that the dipole moment of a pair of chain-ends $\mu_{E,E'}$- is different from the one of ester bonds. The surface potential of the layer is given by the sum of the individual contributions from dipole moments of end-group pairs and repeat units as:

$$\Delta V = \frac{1}{\varepsilon} \frac{(N_B * \mu_B + N_{E,E'} \mu_{E,E'})}{A} = \frac{1}{\varepsilon} (\Gamma_B \mu_B + \mu_{E,E'} \Gamma_{E,E'}) \quad (3)$$

The degradation experiments are carried out under isobaric conditions. It is assumed that at constant degradation surface pressure π_D, the areal concentration of chain segments is constant (Γ_0). The segments can either be repeat units (B) or pairs of chain-ends (E,E').

$$\Gamma_0 = \Gamma_B + \Gamma_{E,E'} \quad (4)$$

By replacing Γ_B in Eq. 3 by Eq. 4, we obtain:

$$\Delta V = \frac{1}{\varepsilon} (\mu_B \Gamma_0 - \mu_B \Gamma_{E,E'} + \mu_{E,E'} \Gamma_{E,E'}) = \frac{1}{\varepsilon} (\mu_B \Gamma_0 + \Gamma_{E,E'} (\mu_{E,E'} - \mu_B)) \quad (5)$$

Under isobaric conditions, the time dependence of the surface potential of the monolayer is described entirely by the time dependence of the areal concentration of end-groups:

$$\Delta V(t) = k_1 + k_2 \Gamma_E(t) \quad (6)$$

Here, $k_1 = \frac{1}{\varepsilon} \mu_B \Gamma_0$, the surface potential of the layer without end-groups (infinite molecular weight) and $k_2 = \frac{1}{\varepsilon} (\mu_{E,E'} - \mu_B)$, the change of the surface potential when one ester bond is converted to a pair of end-groups.

From Eq. 6, we infer that we can analyze the degradation mechanism just by comparing the curves of $\Delta V(t)$ and $A(t)$. The mechanisms discussed here are a) degradation via cuts at the chain-ends and b) degradation via random bond scission (Figure 1).

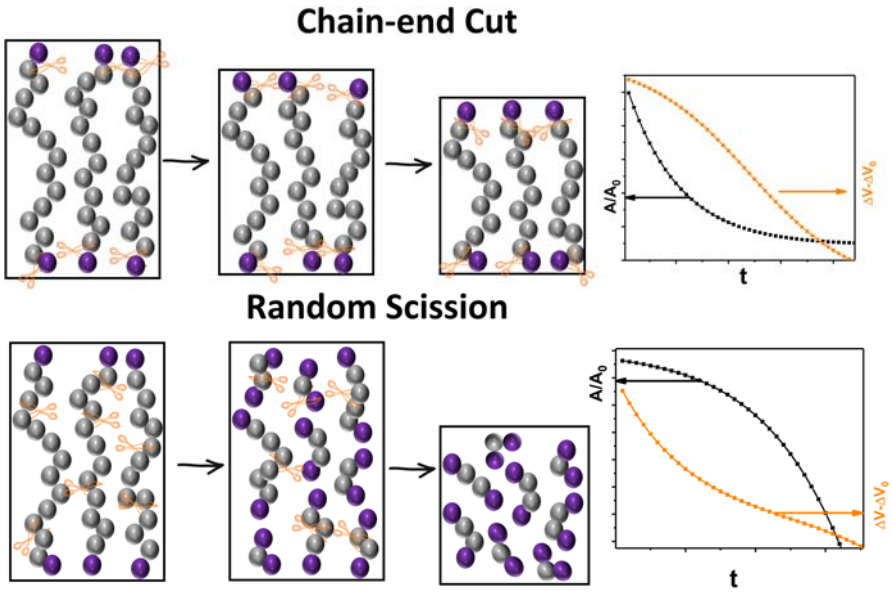

Figure 1: Schematic representation of the evolution of the areal concentration of end-groups when chains degrade via chain-end cuts (top) or a random scission mechanism (bottom).

In the limiting case, where cuts occur exclusively at the chain-end, the end group pairs generated by bond scission are immediately solubilized. Then, the number of chain-ends $N_{E,E'}$ is constant and Γ_E increases solely due to the decrease of the film area. In a plot of $\Delta V(t)$ and $A(t)$ (Figure 1, top), $\Delta V(t)$ decreases slower than $A(t)$. In contrast, in a random scission mechanism, many bonds need to be broken before a considerable number of water-soluble fragments are generated. Many of the end-group pairs generated by bond scission remain in the layer. As a consequence, $N_{E,E'}(t)$ increases and a considerable change of $\Delta V(t)$ at nearly constant $A(t)$ is expected (Figure 1, bottom). This delay is expected to increase with hydrophobicity and molecular weight of the macromolecules.

RESULTS AND DISCUSSION

Surface potential of PHA monolayers

Abiotic degradation of PHA takes place under alkaline conditions [14, 15]. Our previous studies on the monolayer degradation of PHA (co)polyesters have revealed that, when conditions were sufficiently alkaline to result in notably faster degradation, the solvent quality of the A-W interface increases. When compared to water, (co)polyesters, especially PHAs, are relatively nonpolar [17]. Our working hypothesis was that the polarity of the ester bonds increases when hydroxyl ions are forming coordinative bonds with the partially positively charged carbonyl carbon atoms. The surface potential is therefore expected to be dependent on the hydroxyl ion concentration of the subphase.

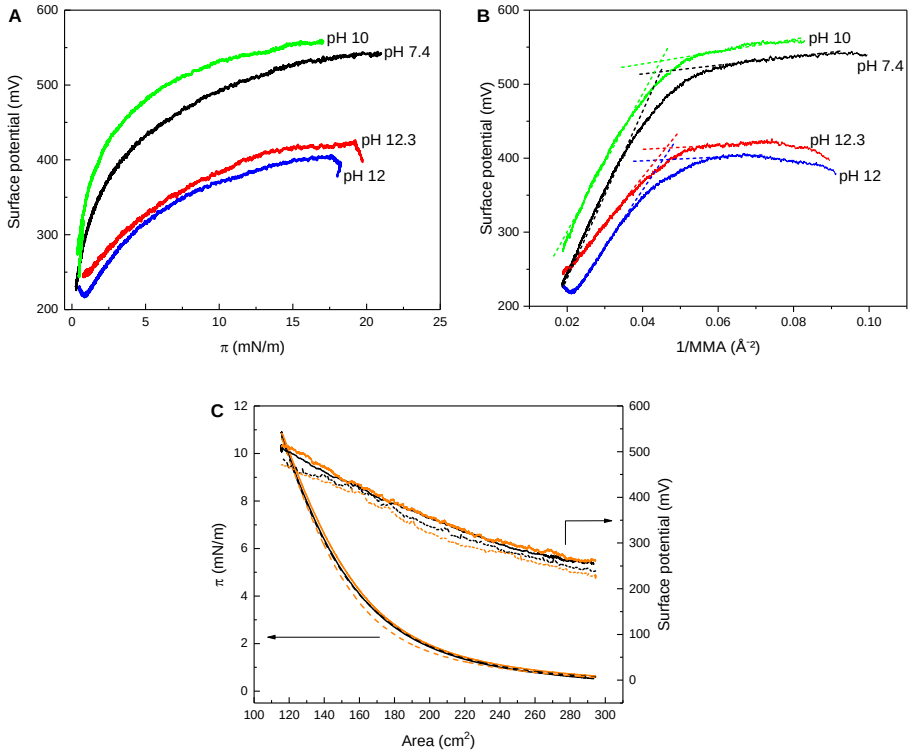

Figure 2. Surface potential of PHB monolayers at different pH (A and B), and during compression-expansion cycles (C). A) Surface potential-surface pressure (ΔV-π) plot of PHB isotherm (pH 7.4 –12.3). B) Plot of surface potential versus inverse of area per repeat unit (ΔV-1/MMA) (pH 7.4 – 12.3). C) Compression-expansion isotherm of PHB at pH = 7.4. Compression cycle 1 (black solid line), expansion cycle 1 (orange solid line), compression cycle 2 (black dashed line), expansion cycle 2 (orange dashed line).

The $\Delta V - \pi$, and $\Delta V - A$ isotherms are shown in Figure 2A and 2B. After spreading the PHB solution at a final concentration of 0.05 µg/ml and reaching a surface pressure of ≈ 0.5 mN/m, the ΔV relative to the bare water was ca. 220 mV in most of the subphases (Figure 2A). When plotting ΔV over 1/MMA according to Eq. (2) (Figure 2B), a linear relation indicates that the dipole moment of the molecules perpendicular to the water surface (μ_z) does not change. For PHB, the linear relation was retained up to a surface pressure of 6 mN/m for PBS and pH = 10 as subphase, and 7.5 mN/m for pH > 10. This surface pressure agrees reasonably well with the transition from the semi-dilute to the concentrated regime for PHB monolayers [12].

According to the Helmholtz equation, the slope of the ΔV vs. Γ is given by $\frac{\mu_z}{\varepsilon}$. The field passes mostly through air, so the permittivity should not be affected by the pH of the solution. Then, the dependence of the initial slope on pH reveals the influence of hydroxyl ions on the dipole moment of the ester bonds. Indeed, differences are observed between pH = 7.4 - 10 and the more alkaline KOH solutions with pH = 12 - 12.3. Figure 2B shows that μ_z is actually lower at pH ≤ 10 than pH = 12 - 12.3. It is worth mentioning that the ΔV values are relative to the bare interface; therefore, they should not be compared in quantitative terms. Furthermore, the surface potential can be related to the surface density by $\mu_z = \Delta V * MMA * \varepsilon$, where the constant $\varepsilon = 8.85\times10^{-12}$ F·m^{-1}. When the

component of the molecular dipole moment perpendicular to the water surface μ_z is calculated from the slope of the linear regime of ΔV vs $\frac{1}{MMA}$ (Figure 2B), the dipole moment per unit is 290 mD at pH = 7.4 – 10 and 170 mD at pH = 12 – 12.3. These results indicate that the ester bond contribution decreases with increased pH, which might be related to the solvent quality of the subphase. At pH < 10, PHB is under relatively bad solvent conditions at the air water interface, while pH > 12 becomes a better solvent in which the polymer chains are rather flexible and less oriented [12].

As the compression continues above the phase transition of the monolayers, the decrease of the slope indicates saturation of the interface due to a closed packing of the PHB chains. Previous work from our group reported that PHB monolayers do not collapse up to 5 Å^2 per molecule. Conversely, the $\Delta A - \pi$ isotherms of PHB showed a distinct kink around 12 mN/m, which shifted to higher surface pressure when the pH increased. This kink has been defined as the 2D to 3D transition of the monolayers, based on the interfacial characterization of the films by infrared spectroscopy, rheology, and Brewster angle microscopy techniques [12, 17].

In order to detect alterations of the surface potential by degradation, it is beneficial to work under conditions where the surface potential is most strongly affected by alterations of the areal concentration of repeat units. This condition is given by the maximum of the slope of the surface potential vs. surface pressure or MMA. For PHB at the designated pH for degradation of 12.3, this was observed at π = 4 mN/m. No hysteresis effect on the surface potential was observed up to 12 mN/m (Figure 2C), the 2D to 3D transition of PHB monolayers.

Variation of PHB dipole moment during hydrolytic degradation

The evolution of the surface potential in Langmuir monolayers under isobaric conditions detects the generation of end-groups by the cleavage of bonds in the backbone according to Eq. (6). The molecular degradation mechanism of PHB monolayers could be studied using this approach and the variation on the polymer molecular weight during degradation could be deduced. The decrease in the area and the progress of the surface potential of PHB monolayers were simultaneously measured on PBS as subphase. The initial surface potential ΔV = 460 was recorded for 10 min at surface pressure 4.0 mN/m and turned out to be constant. Then, 2 mL of KOH 10 M was injected under the subphase to obtain a pH of 12.3-13. The A/A_0-time plot (Figure 3) initially shows a fast solubilization of the spread polymer, consistent with a chain-end scission model (Figure 1, top). This fast initial degradation diverges from a random scission process, expected for the enzymatic mediated hydrolysis of PHA [9]. The film area decreases in parallel with the monolayer surface potential. The area reduction rate decreases after about 400 min, while the surface potential reaches its zero point (subphase surface potential). Continuous measurements of the pH during degradation experiments have shown a decrease of the subphase pH due to dissolution of CO_2. The inset in Figure 3 shows that the contribution of the decrease in the surface potential due to the shift in the pH is about -100 mV after 300 min. A comparison of the observed (Figure 3) and the expected shifts (Figure 1) between the A(t) and ΔV(t) curves indicates that the degradation mechanism is neither an exclusive chain-end cut, nor a random scission mechanism, but somewhere in between.

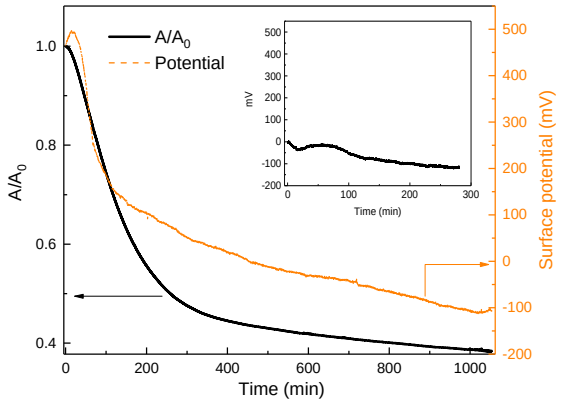

Figure 3. Decrease of the surface area (A/A_0, solid black line), and surface potential (ΔV, dotted orange line) with time for monolayers of PHB after injection of 10 M KOH. Inset: negative control of surface potential ΔV(t) of the bare KOH subphase with pH = 12.3.

Variation of PHOHHX dipole moment during enzymatic degradation

Degradation experiments under enzymatic catalysis were performed by using PBS as a subphase (pH = 7.4) together with $PhaZ_{sex2}$, which was injected under the preformed films at C_f = 0.2 µg/ml in the subphase of the Langmuir trough. When the film was held at a surface pressure of 8 mN/m, the surface potential decreased very rapidly (Figure 4), while the surface area remained constant. Only when the surface pressure was increased to 10 mN/m, the surface area started to decrease. As expected for enzymatic hydrolysis of (co)polyesters, the sigmoid shape of the A/A_0 curve suggested a random scission of cleavable ester bonds. In a random-scission mechanism, the generation of a significant number of water-soluble fragments takes a certain time for this polymer, resulting in an initial period of slow dissolution. In this period, the surface potential decreases rapidly due to the new chain-ends produced by the hydrolysis of the ester bonds. The final value of the surface potential was achieved at about 50% dissolution. The change of the surface potential is caused by an increase of the areal concentration of end-groups. This concentration is closely related to the molecular weight of the chains. During degradation, the molecular weight reaches an asymptotic value close to the solubility limit, since further chain cuts result in direct solubilization of fragments. Hence, it is clear that the asymptotic behavior of the surface potential corresponds to the asymptotic decrease of the molecular weight during degradation. The instability of the dipole moment, seen by a fluctuation in the measurement may be due to the formation of PhaZ-PHOHHx complexes affecting the surface potential measurement. This observation is also based on the fact that PHA depolymerases are able to bind to PHA granules accumulated in the bacterial cytoplasm. The control with the enzyme alone showed a decrease in the surface potential of -100 mV.

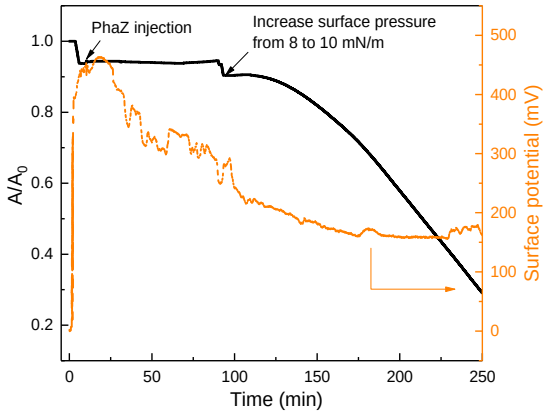

Figure 4. Decrease of the surface area (A/A_0, solid black line), and surface potential (ΔV, dotted orange line) with time for monolayers of PHOHHx after injection of $PhaZ_{Sex2}$ at a final concentration of 0.2 µg/ml in the PBS (pH = 7.4) subphase.

CONCLUSIONS

The interdependency between the surface potential and the degradation behavior of PHB and PHOHHx (co)polyesters was investigated at the air-water interface based on two degradation scenarios. The change of the area per molecule at the interface gives information to the generation of soluble degradation products, while the shift in the surface potential of the ester bonds shows the change of the dipole moment due to conversion of ester bonds into chain-ends. The dipole moment of the monolayers depended on the concentration of the hydroxyl ions. By combining two experimental approaches - hydrolytic degradation by base attack and enzymatic mediated degradation - with a theoretical model, it was possible to link the surface potential decrease to the specific degradation mechanisms. This approach can be used to predict the evolution of the surface potential of polyester-based biomedical devices, in a simple, fast and controlled way. Further efforts will be made to enhance the theoretical model to directly deduce the evolution of the molecular weight to the evolution of the surface potential. To determine k_1, the surface potential of samples with different molecular weights needs to be measured. To gain a more precise understanding of k_2, the influence of surface charges and the electric double layer on the surface potential has to be considered.

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