Use of *In-situ* Atomic Force Microscopy to Monitor the Biodegradation of Polyhydroxyalkanoates (PHAs)

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SUMMARY: Thin films of a co-polymer mixture of poly 3-hydroxybutyrate and poly 3-hydroxyvalerate P(3HB-3HV) were spun-cast onto glass slides resulting in 35 nm thick layers with a spherulitic microstructure. An untyped strain of S*treptomyces sp.* bacteria was isolated from soil samples, and it's PHA depolymerase was used to degrade the P(3HB-3HV) thin films. Both *ex-situ* and *in-situ* atomic force microscopy (AFM) biodegradation studies were performed to determine the kinetics of the biodegradation over the course of three hours at room temperature. *Ex-situ* AFM was performed in Tapping Mode and *in-situ* AFM was performed in the PHA depolymerase using contact mode AFM in the liquid cell, allowing for the real-time analysis of P(3HB-3HV) biodegradation. Biodegradation is observed uniformly throughout the surface, and can be observed within 30 min. of depolymerase exposure. *In-situ* AFM analysis yields a linear degradation rate as a function of time, while the *ex-situ* study suggests a more complex kinetics.

Introduction

Polyhydroxyalkanoates (PHAs) are a family of biodegradable polymers that are being considered in commercially manufactured plastics. PHAs are produced by a variety of bacteria as an energy storage media when carbon levels are high and other necessary nutrients such as nitrogen, phosphate, and sulfur are low. PHAs are of considerable technological interest because they can be processed to produce plastic materials that are fully biodegradable in soil, water, and sewer environments [1,2]. Another advantage of PHAs is they can be synthesized from entirely renewable resources. Commercial production of PHAs is based on sugars and fatty acids as carbon sources. PHA depolymerases produced by soil and water microbes degrade reduces PHAs back to CO_2 and water. Thus, one can envision a plastic production that relies on entirely renewable resources rather then the finite fossil fuel

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used in petrochemical-based plastics [3]. The general chemical structure for PHAs is shown in Figure 1. The most studied form of PHA is polyhydroxybutyrate, P(3HB), in which the R substituent is a methyl group. Another common structure is polyhydroxyvalerate, P(3HV), in which the R substituent is an ethyl group. In this study, we have examined co-polymer blends of P(3HB-3HV).

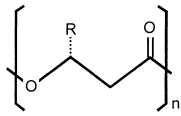

***Figure 1**: Chemical structure of PHAs.*

There have been many studies on the biodegradation of bulk PHAs during the past decade [4-12], and several review articles on the biodegradation process are in the literature [1,13,14]. Many different analytical techniques have been reported in these studies to characterize the biodegradation process including scanning electron microscopy (SEM) [5,10], optical microscopy [10], transmission electron microscopy (TEM) [15], gas chromatography [7], and nuclear magnetic resonance (NMR) [16,17] among others. What most of these studies have in common is that the biodegradation has been performed on bulk samples, and almost all of these studies have been carried out in an *ex-situ* manner, meaning the degradation process and the characterization process are performed as separate experiments. In these studies, the depolymerase has been either isolated and purified from bacteria known to degrade PHAs, or the polymer has been placed in a soil, seawater, or sewer environment and allowed to degrade over a weeks to months time period. From this starting point, degradation rates have been determined via mass loss of the bulk sample (See for example: Tomasi, *et al.* [10]). While this method of determining the degradation rate is important to characterize the bulk degradation process, it does not provide a direct observation of either the degradation rate or the initial stages of depolymerization. Thus, an *ex-situ* study does not allow for a real-time understanding of the changes in microstructure or materials properties that can be achieved through an *in-situ* study.

We report an atomic force microscopy (AFM) study to follow changes in surface microstructure as a function of degradation time *in-situ* and in real time. The AFM produces a three-dimensional topographic image of the surface with cubic nanometer resolution. PHB has a density of 1.18 g/cm^2 [18]. Using it as an example, the best mass measurements can be made

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only to the microgram (10^{-6} g) level. AFM can easily resolve 1.0 μm^3 features, thus weighing about one picogram (10^{-12} g). This suggests that AFM has the potential to be many orders of magnitude more sensitive to the initial stages of degradation than bulk mass measurements. The AFM can also be used to monitor the surface microstructure in thin films of PHAs in an enzymatic environment using the liquid cell capability of AFM.

In the vast literature on the preparation and characterization of PHAs, to our knowledge there have been no reports of *in-situ* biodegradation and microscopy of these materials. However, there have been several analogous studies on the *in-situ* degradation of other polymer systems. The most notable example of this is a series of studies reported by Shakesheff and co-workers at Nottingham [19-21]. In these papers, the authors reported on the *in-situ* AFM analysis of the aqueous degradation of poly(sebacic anhydride) and poly(DL-lactic acid) copolymer blends. In particular, the authors demonstrated the effect of pH on degradation using the liquid cell that was not possible with other high resolution microscopy techniques such as SEM or TEM. In another report, a biodegradable ceramic/polymer composite was produced and the degradation was studied via AFM [22]. In this study, poly (ε-caprilactone) was terminated with sol-gel prepared triethoxysilane making a biocompatible and biodegradable nanocomposite. It should be noted, that the degradation described in the former reports [19-21] was not enzyme based, but based on the hydrolysis of the polymer in an aqueous environment as one would encounter in a drug delivery system, and the degradation reported in the latter studies [22] was examined *ex-situ* in an enzymatic environment. However, both studies illustrate the power of the AFM to study biodegradation of polymer surfaces. *In-situ* AFM has also recently been employed to directly observe the growth of the spherulitic structure in thin films of P(3HB-3HV) copolymer blends [23]. Using the phase-imaging technique [24], the lamellar structure of the growth front of the spherulitic microstructure was directly observed, and kinetics of spherulite formation was reported. Each of these studies illustrates the potential for AFM as a characterization tool for studying the biodegradation of polymer materials *in-situ* and in real time.

Experimental

Isolation and Identification of PHA-Degrading Bacteria

PHA granules were obtained from cultures of *Ralstonia eutropha H16* as described [25], except that 0.2% fructose was used as the carbon source. PHA agar plates were prepared by overlaying 20 mL of a mineral salts agar medium [26] with 5 mL of the same medium

containing 0.1% w/v PHA granules. A sample of soil (1 g) was diluted with 100 mL of sterile 0.85% saline solution and shaken vigorously for 1 min. The suspension was allowed to settle for 1 min., and 100 μL aliquots of the supernatant liquid were spread onto the surface of PHA agar plates. The plates were then incubated for several days at 30°C to allow for bacterial growth. Bacterial colonies which formed clearing zones in the opaque PHA layer of the plates were picked off with sterile toothpicks and streaked for isolation onto fresh PHA agar plates. Two selected isolates were identified by membrane fatty acid analysis (Microbial ID, Inc., Newark, DE) as an uncharacterized species of *Streptomyces* (denoted *Streptomyces spp. 5A*). The isolates were subsequently maintained on nutrient agar slants and plates.

PHA Depolymerase Preparation

Cells of *Streptomyces spp. 5A* were grown in 50 mL of nutrient broth for 16 hr. at 30°C with shaking. The cells were then harvested aseptically by centrifugation, resuspended in 50 mL of mineral salts liquid medium [26], and transferred to a 4-liter Erlenmeyer flask containing 1 liter of the same medium with 0.2% w/v PHA granules. The culture was incubated with shaking for 2-3 days at 25°C, after which time degradation of the PHA granules was visibly complete. The culture was centrifuged to remove cell material. Solid ammonium sulfate was added to the supernatant solution to bring it to 70% saturation of ammonium sulfate at 0°C. The solution was stirred gently on ice, and pH was maintained at about 7.5 by addition of 1 M Tris-Cl buffer, pH 8.0. The solution was centrifuged at 20,000 x *g* for 20 min. at 4.0°C. The pellet was redissolved in 25 mM Tris-Cl, pH 7.5 containing 1 mM $CaCl_2$. Particulates were removed by passing the enzyme through a syringe filter (pore size, 0.45 μm). The enzyme was stored at -20°C until use.

Depolymerase activity was determined by spotting 2 μL aliquots of the enzyme onto the surface of glass slides overlaid with 1.0 % w/v agarose containing 100 mM Tris-Cl (pH 7.9), 1 mM $CaCl_2$, and 0.1 % w/v PHA. The slides were incubated at 37°C until clearing zones were visible. The diameter of the clearing zone was a semiquantitative measure of the enzyme activity.

Thin Film Formation

P(3HB-3HV) thin films were deposited onto glass microscope slides by spin casting from solutions using a commercial spin deposition system. Glass slides were cleaned in a three step

process of acetone followed by isopropyl alcohol then deionized water. Each cleaning step was performed for 5 min. under ultrasonic agitation. The slides were thoroughly dried with compressed N_2 between each cleaning step. Solutions of the co-polymer PHB and 14% PHV by weight (Aldrich) P(3HB-14%-3HV) were prepared in chloroform with concentrations ranging from 1.0 to 8.0 mg/mL. Through calibration of the spin-cast apparatus, thin films can be made with a uniform thickness across a 1 x 1 inch glass substrate. A spin speed of 650 - 700 rpm results in a thickness of approximately 35 nm for a 6 mg/mL solution of P(HB-14%-HV) which was determined using stylus profilometry in a masked region of the slide. The results in the remainder of this report are all for 6 mg/mL solutions of the P(3HB-14%-3HV).

Biodegradation Studies

The *ex-situ* biodegradation experiments were performed as follows: a drop of concentrated *Streptomyces* PHA depolymerase was placed onto the surface of the P(3HB-3HV) thin films, and these were covered with a glass coverslip. Samples were taken at half-hour exposures for up to 3 hr., rinsed in deionized water, dried, and imaged in the AFM. For the *in-situ* studies, the thin film P(3HB-3HV) samples were placed in the AFM, and the filtered *Streptomyces* enzyme solution was added to the liquid cell. AFM scans of the same location on the sample were taken at 1, 1.5, 1.75, and 2 hrs. When the sample was imaged in the depolymerase enzyme solution, small particles left in the buffer solution after filtering adhered to the cantilever causing scan line errors in the AFM images. It is known that enzyme adhesion to the cantilever is problematic when imaging in an enzymatic solution [27]. In order to prevent this image artifact, the enzyme solution was allowed to equilibrate for 15 min. before any images were taken so all the images in the *in-situ* series have a consistent probe. All reactions took place at room temperature for both *ex-situ* and *in-situ* experiments. It should be noted that the *Streptomyces* enzyme decreased in activity before 6 hours at room temperature and no further degradation occurred. This is presumably due to the denaturing of the enzyme.

AFM was performed on a Digital Instruments (DI) NanoScope III Extended Multimode instrument. For the *ex-situ* experiments, height images were taken of the samples in Tapping Mode at a scan rate of 1.80 Hz using standard Si Tapping Mode cantilevers (Olympus). For the *in-situ* experiments, height images were taken of the samples using a liquid cell in contact mode using standard Si_3N_4 contact mode cantilevers at a scan rate of 1.80 Hz. Scan sizes ranged from 10 to 50 μm^2. A first order flatten was performed on each sample after

acquisition. Quantification of the AFM data was performed using bearing, RMS roughness, and cross sectional analyses available with the DI analysis software.

Results and Discussion

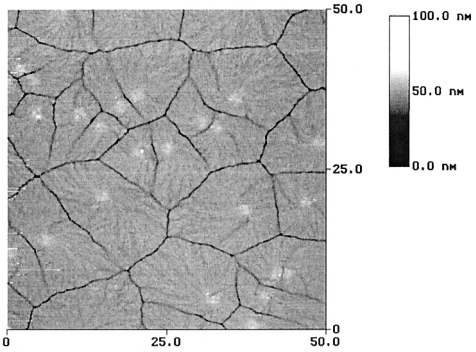

Figure 2: *AFM image (50.0 μm scan) of P(3HB-3HV) thin film as-deposited in Tapping Mode in air.*

The thin films of P(3HB-3HV) exhibited spherulitic microstructures approximately 5-7 μm in diameter distributed uniformly across the surface as-deposited (see Figure 2). This microstructure is widely known for semicrystalline polymers above the glass transition temperature (T_g for PHB ~ 2°C). PHB is a predominantly crystalline polymer while PHV is predominantly amorphous at room temperature.

Ex-situ degradation using the *Streptomyces* PHA depolymerase exhibited rapid signs of biodegradation with depolymerization evident after only 0.5 hrs. occurring evenly across the polymer surfaces. Figure 3 (A – F) shows a series of AFM images taken *ex-situ* with P(3HB-14%-3HV) thin films exposed to the *Streptomyces* depolymerase ranging in time from as-deposited (0 hr.) to 3 hrs. Degradation is clearly evident in the dark pits distributed uniformly throughout the spherulites. As degradation increases, these etched regions are larger and more numerous giving the surface a rougher appearance. By the end of three hours (Figure 3 (F)), the films have degraded completely through the polymer in some regions down to the glass substrate. This is shown in a cross-section of Figure 3 (F) in Figure 4. One can clearly see a flat surface 29.4 nm below the film surface of the underlying glass substrate, which is the same thickness determined through the prior calibration of the films.

The data from Figure 3 was analyzed using both bearing analysis and RMS roughness [28,29] and is shown in Figures 5 and 6, respectively. Bearing percent area was determined using the threshold feature in the DI analysis software. Briefly, the bearing percent area is an analysis commonly used for such processes as semiconductor etching. The bearing percent area is a reflection of the percentage of data points above a given threshold. For example, at 0%

bearing area, every x, y, z data point is below the threshold value, while at 100% bearing area every data point is above the threshold value. A cursor can be moved to indicate which data points on the image are included above the threshold. Using the threshold method, the bearing cursor was moved such that regions that were degraded (potentially including the spherulite boundaries) were not highlighted, while all of the data points above the cursor were illuminated. The percent bearing area was then subtracted from 100% to indicate the percentage of data points below the threshold plane. This is plotted as a function of time in Figure 5. Ideally, this analysis is performed on flat substrates such as semiconductor surfaces that are being etched because the roughness introduces considerable variability in the data. We are currently developing a scheme to overcome the initial roughness of the spherulitic surface.

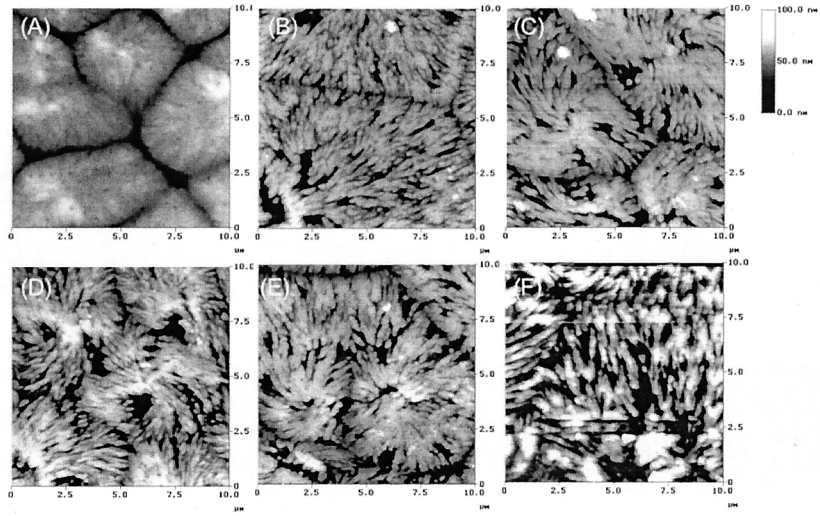

Figure 3 *Tapping Mode AFM images (10 μm scan, 100 nm z-range) of P(3HB-14%-3HV) thin films after ex-situ biodegradation in Streptomyces depolymerase at room temperature. Exposure times are as follows (A) as-deposited, (B) 0.5 hr., (C) 1.0 hr., (D) 1.5 hr., (E) 2.0 hr., (F) 3.0 hr.*

There is an increase in degraded area for the first 2 hours, followed by a rapid increase observed from 2 to 3 hours. We have repeatedly observed this behavior, and it is currently the subject of further investigation. The RMS roughness was also calculated over the three hour degradation period and shown in Figure 6. Again, a two-fold increase in the surface

roughness is seen as a function of time. A limitation of using bearing analysis for quantification of this data is that unlike in a semiconductor etching process, where the substrate is extremely flat in the initial stages, the percentage of the image occupied by spherulites is not the same in each image. These represent a significant fraction of "low" data points on the image. While this artifact cannot be eliminated, using the exact same scan size should have approximately the same percentage of spherulites for the given surface area. This needs to be investigated further to ensure that this assumption is valid.

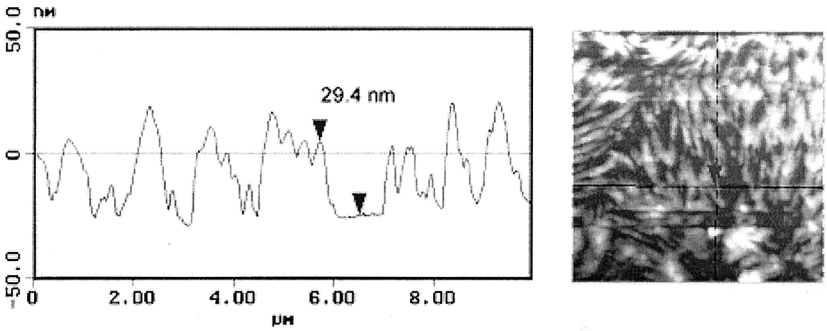

Figure 4 *Cross-sectional analysis of Figure 3 (F). P(3HB-3HV) thin film is degraded to the surface of the glass substrate as indicated by the flat surface in the right arrow. Film thickness is 29.4 nm.*

Although Figures 3-6 clearly demonstrate biodegradation over time, the data has a wide statistical variation associated with the deposition of several different P(3HB-3HV) thin films, different sampling areas on the surfaces, and the spherulitic microstructures. *In-situ* AFM measurements are one way to alleviate this problem. By using a constant sampling area and measuring a single spot on the sample over a period of time, the variations in sample deposition are eliminated.

In-situ degradation using the liquid cell proved to have a much narrower range of values than the *ex-situ* data. Visible degradation was observed in all of the *in-situ* samples as shown in Figure 7 (A - C). For example, note the degraded region in the lower right-hand corner of each image indicated with an arrow. In Figure 7 (A), the sample degraded for 1 hour, and the width of this feature is 0.684 nm. In Figure 7 (B) a 1.5 hr. exposure results in a 0.919 nm feature, and in Figure 7 (C) at 2 hrs. of exposure it is 1.094 nm wide. *In-situ* AFM analysis offers the advantage of being able to identify one structure on the polymer surface and monitor the degradation of that specific feature.

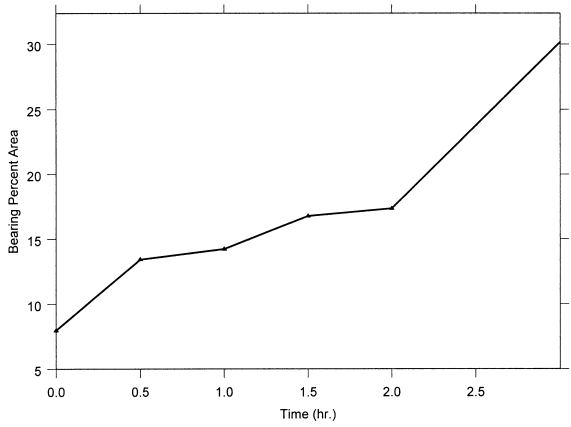

Figure 5 *Bearing Analysis of Figures 3 (A-F)*

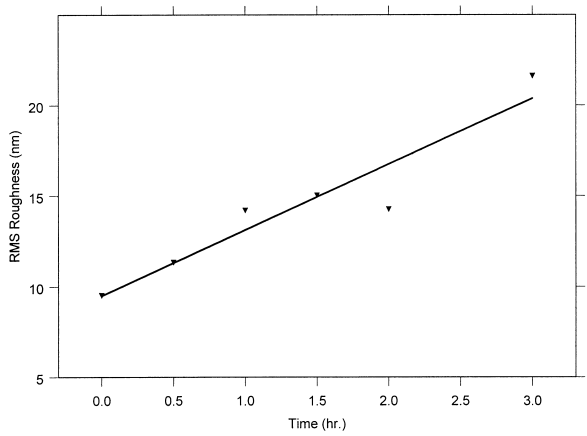

Figure 6 *RMS (R_q) analysis of Figures 3 (A-F)*

The bearing analysis of the *in-situ* images was performed on several regions within each image not effected by scan line errors. These showed a similar increasing trend in the percent area below the threshold plane as shown in Figure 8. This decrease in bearing area closely correlates to a linear degradation by the *Streptomyces* depolymerase. This technique may allow predictions to be made about the nature of P(3HB-3HV) degradation in a *Streptomyces* enzyme environment.

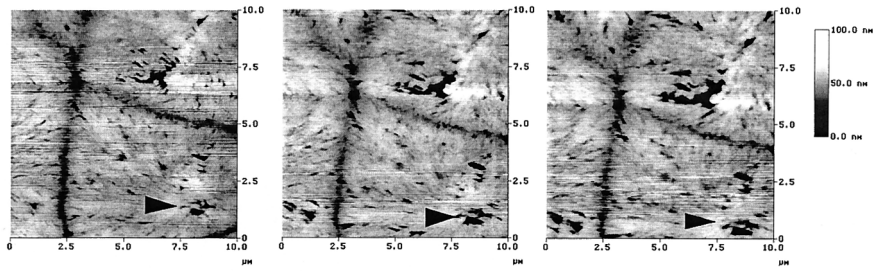

Figure 7: *AFM images (10 μm scan) in contact mode of P(3HB-3HV) thin films after (A) 1 hr. (B) 1.5 hr. and (C) 2 hr. exposure to Strepomyces depolymerase. Images were taken in-situ in the enzyme solution using the liquid cell. Arrow indicates a common feature in each image.*

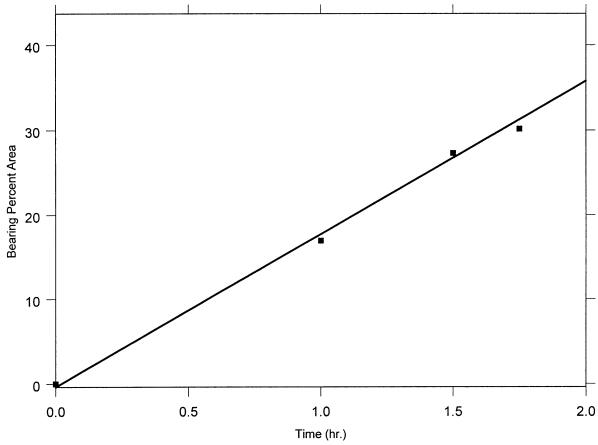

Figure 8 *Bearing percent area below a threshold plane from the data from Figure 7. Samples were taken from different areas of the same P(3HB-3HV) image and averaged.*

We note a difference in biodegradation rates in the *ex-situ* and the *in-situ* degraded films with the *ex-situ* degrading more rapidly. The most likely cause of this behavior is that these were performed at different times with a different strain and purity of *Streptomyces* depolymerase. The biodegradation rate is closely correlated with the purity and concentration of the depolymerase. In the *ex-situ* study, a concentrated drop of the depolymerase was placed directly onto the surface and covered with a cover-slip. Thus a lot of surface area was exposed to a high concentration of the depolymerase. In the *in-situ* study, the depolymerase

was filtered and diluted slightly in buffer to minimize the effects of probe contamination by the enzyme. While this is the most likely source of the different biodegradation rates in the two experiments, it remains a possibility that the force of a scanned probe across the surface is effecting the degradation rate. It remains a possibility that the force applied in contact mode in the *in-situ* study may alter slightly the orientation of the surface, thus hindering the enzymatic attack of the surface and needs to be verified.

We have performed high-resolution phase imaging analysis of these films and observed a lamellar structure with lamellae on the order of 10 – 12 nm within the spherulites [30]. We believe that the contrast in the phase image may be due to the interpenetration of amorphous PHV and semi-crystalline PHB regions. We are currently studying whether the biodegradation occurs preferentially in the amorphous or crystalline regions, or whether the degradation is uniform in both regions. Any complimentary surface analysis techniques would need to have 10 nm spatial resolution in order to observe this phenomena, and AFM is particularly well suited to study this.

Conclusion

We have used AFM to monitor the biodegradation of P(3HB-3HV) thin films using AFM both *ex-situ* and *in-situ* in a *Streptomyces sp.* produced PHA depolymerase enzyme solution. Bearing analysis and roughness confirm a steady progression of degradation as a function of time. Data from the *in-situ* study suggests that the initial stages of biodegradation is a linear function, while the *ex-situ* data suggests that there may be a more complicated relationship with a slower initial rate followed by a faster rate as the surface is opened up by the enzyme attack. Future studies are ongoing to better understand the kinetics of the initial stages of enzymatic biodegradation of these materials. This study also demonstrates the potential of *in-situ* liquid cell AFM to monitor in real time dynamic biological processes.

Acknowledgements

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