6836 *Macromolecules* **2009**, *42*, 6836–6839
DOI: 10.1021/ma901481h

Macromolecules
COMMUNICATION TO THE EDITOR

Embedded Enzymatic Biomaterial Degradation

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Received July 8, 2009
Revised Manuscript Received August 13, 2009

Introduction. Conventional thinking when designing bioresorbable materials is to use the intrinsic properties of synthesized materials to regulate their degradation rate. For example, considerable effort has been dedicated to introducing comonomers to manipulate the bioresorption rate of PCL.[1] While this is a well-established strategy, each copolymer composition represents a new medical material that must be assessed by an array of expensive tests requiring years. Herein, we describe a simple but powerful alternative approach to control biomaterial lifetime using PCL as the model system. The hypothesis tested is that biomaterials bioresorption rate can be regulated by embedding an enzyme within a bioresorbable polymer matrix when that enzyme is active for matrix hydrolysis. The PCL-degrading enzyme, *Candida antarctica* Lipase B (CALB), was selected and surfactant paired to render CALB soluble for dispersion within a PCL matrix. This principle termed *embedded enzymatic degradation* can be used for any biomaterial for which an enzyme is known or can be developed for matrix hydrolysis. Therefore, it represents a general approach to be deployed to control biomaterial lifetime.

PCL is an excellent candidate for controlled release and other biomaterial applications due to its availability, biodegradability, ease by which it can be melt-processed into various shaped substances, biocompatibility, and miscibility with many drugs.[2] PCL is a soft material that has a peak melting temperature, tensile stress at break, elongation at break, and tensile modulus of 60 °C, 4 MPa, 800−1000%, and 386 MPa, respectively.[3] However, numerous studies have shown that PCL degrades slowly when implanted in animals. For example, PCL capsules, implanted in rats, with initial molecular weight (M_w) 66 000, retained their shape for over 2 years.[2] By 30 months, PCL capsules broke into low molecular weight pieces (M_w 8000).[2] The slow degradability of PCL limits its use for systems that require shorter lifetimes.[1] Nevertheless, PCL and its copolymers continue to be intensely studied for a range of medical applications.[4]

Herein, surfactant-paired CALB was mixed in toluene with PCL and films were prepared by solution casting. Sodium bis(2-ethylhexyl)sulfosuccinate (AOT), the surfactant used to solubilize CALB, is approved for administration to both humans and animals[5] and was found to be safe for encapsulation and delivery of lipophilic drugs in aqueous based delivery systems.[6,7] Biocompatibility experiments of CALB are currently under progress. If CALB biocompatibility is not suitable, methods are available to identify and remove segments in proteins that trigger an immune response.[8] Another alternative currently under study is exploring other enzymes that have high homology to those naturally found in animal systems and that also catalyze PCL hydrolysis.[9]

CALB was surfactant paired with AOT to enable enzyme transfer from the aqueous to the organic phase. The method of ion-pairing is described in the Supporting Information. Ion-paired CALB was embedded in PCL matrices with enzyme contents of 6.5 and 1.65% (w/w). Methods in the Supporting Information describe details by which PCL-embedded CALB films were incubated during degradation studies. Weight loss was determined gravimetrically, and protein content was quantified using the micro-BCA protein assay. Molecular weights were determined at room temperature by gel permeation chromatography (GPC). Scanning electron micrographs were generated with an Amray 1910 SEM and Schottkey field emission gun at 5 kV. DSC measurements were performed using a Perkin-Elmer DSC system with a scan rate of 10 °C/min. CALB was conjugated with fluorescein isothiocyanate (FITC), and this conjugate was embedded in PCL matrices. Imaging of CALB distribution within PCL was performed by confocal microscopy (Leica Microsystems, Bannockburn, IL) using a 488 nm excitation laser.

Details of all procedures and experiments are provided in the Methods section of the Supporting Information.

Results and Discussion. Films with defined quantities of AOT-paired CALB dispersed within a PCL matrix were prepared by solution casting from toluene. Initial surface area, thickness, and average weight of films were 4 cm^2, 80 ± 5 μm, and 37 ± 2 mg, respectively. Concentrations of CALB-AOT investigated herein are 19.4 and 5% (w/w to PCL), which corresponds to CALB contents of 6.5 and 1.6%, respectively. Films were incubated in phosphate buffer solution (20 mM, pH 7.10) with shaking (200 rpm) at 37 °C. Media were replaced with fresh buffer at regular time intervals described below. Figure 1a shows PCL films with 6.5% CALB degraded rapidly, so that by 24 h complete film weight loss was achieved. CALB diffusion from films into the media was determined by micro-BCA analysis. By 2 h, film weight loss and diffusion of CALB into the media reached 48 ± 10% and 18 ± 5%, respectively. Thereafter, while film weight loss increased to 70% by 6 h, CALB release remained unchanged. Thus, by 8 h, while total film weight loss was 70 ± 10%, 80 ± 5% of CALB still remained in films. Surprisingly, from 6 to 16 h, no further film weight loss occurred, but protein diffusion into the media increased to 50 ± 5%. Possibly, film degradation slows due to denaturation of enzymes at surfaces and that, between 8 and 16 h, denatured enzyme at surfaces is replaced with "fresh" CALB from aggregates embedded in films (see confocal analysis studies below).

By reducing CALB content from 6.5 to 1.6%, the time required for complete film degradation was increased from 1 day to about 17 days (Figure 1b). While a large fraction (40%) of film weight loss occurred during the first day, further film weight loss occurred slowly over the following 16 days. Similar to experiments performed with 6.5% CALB, 1.6% CALB also experienced the phenomena where film weight loss remained constant while a large fraction of CALB diffused from films into the media. For 1.6% CALB,

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 Published on Web 08/21/2009

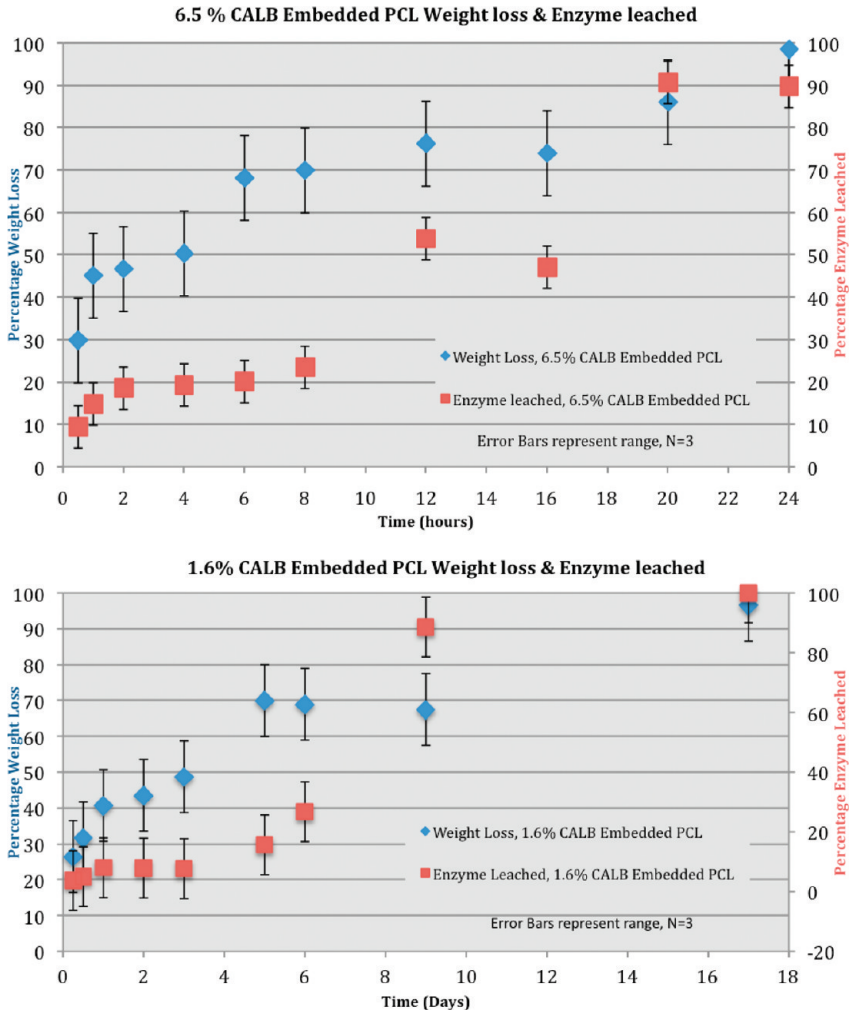

Figure 1. Weight loss and enzyme leached: (a) 6.5% CALB-embedded PCL; (b) 1.6% CALB-embedded PCL.

this occurred between 5 and 9 days while CALB depletion from films increased from 15 to 90 ± 10%.

GPC was used to determine the progression of changes in PCL molecular weight as a function of incubation time for two systems: (i) enzyme added externally to the polymer matrix and (ii) 6.5 and 1.6% CALB embedded in the PCL matrix. Plots of M_n vs % weight loss and polydispersity (PDI, M_w/M_n) are shown in Table 1. Referring to Figure 1, film weight loss reached 30 and 40% early during incubations with 6.5 and 1.6% embedded CALB, respectively. These weight losses were accompanied by large decreases in M_n from 120 000 to about 40 000. Furthermore, PDI increased from 1.7 to about 4.0. For the 1.6% CALB−PCL system, M_n and PDI remained at 25 000−35 000 and 3.5−4.3 until 80% weight loss. These changes in M_n and PDI are explained by CALB degradation of PCL in CALB-embedded films from many surfaces created at numerous interfaces between CALB−AOT aggregates (see below) and the PCL matrix. CALB degradation of PCL occurs by endo attack,[10] creating a fraction of lower molecular weight chains that are trapped within a PCL matrix for which a substantial fraction of the material has not yet been accessed by enzyme and, therefore, remains at its original molecular weight (see GPC traces in Supporting Information Figures s1, s2, and s3). As time progresses during incubations, lower M_n PCL chains are degraded to water-soluble products that diffuse from the matrix and cause weight loss while new oligomers are generated, thereby causing M_n to remain low and PDI to remain high. In contrast, when CALB is added externally to PCL films without embedded enzyme, M_n and PDI remain constant as film weight loss proceeds[11] (Table 1). This remarkable contrast in molecular weight change between embedded and externally added CALB is due to that when CALB is added externally, CALB can only access PCL

Table 1. Variation of Number Average Molecular Weight, M_n, and Polydispersity Index, PDI, with Respect to Percentage Degradation

sample	weight loss (%)	M_n	M_w/M_n
6.5%[a]	0	117 000	1.8
6.5%[a]	30	36 400	3.7
6.5%[a]	60	31 800	3.6
6.5%[a]	80	24 700	5.2
1.6%[a]	0	126 000	1.7
1.6%[a]	30	40 000	4.1
1.6%[a]	60	34 000	4.0
1.6%[a]	80	33 200	4.3
external addition[b]	0	124 000	1.7
external addition[b]	30	128 000	1.7
external addition[b]	60	123 000	1.7
external addition[b]	80	117 000	1.8

[a] CALB embedded within PCL film. [b] CALB added to medium instead of embedding within films. [c] M_n and M_w/M_n, determined by GPC, are number-average molecular weight and polydispersity (PDI), respectively (M_n, error ±5%, and M_w/M_n, error ±5%, represents range $n = 3$).

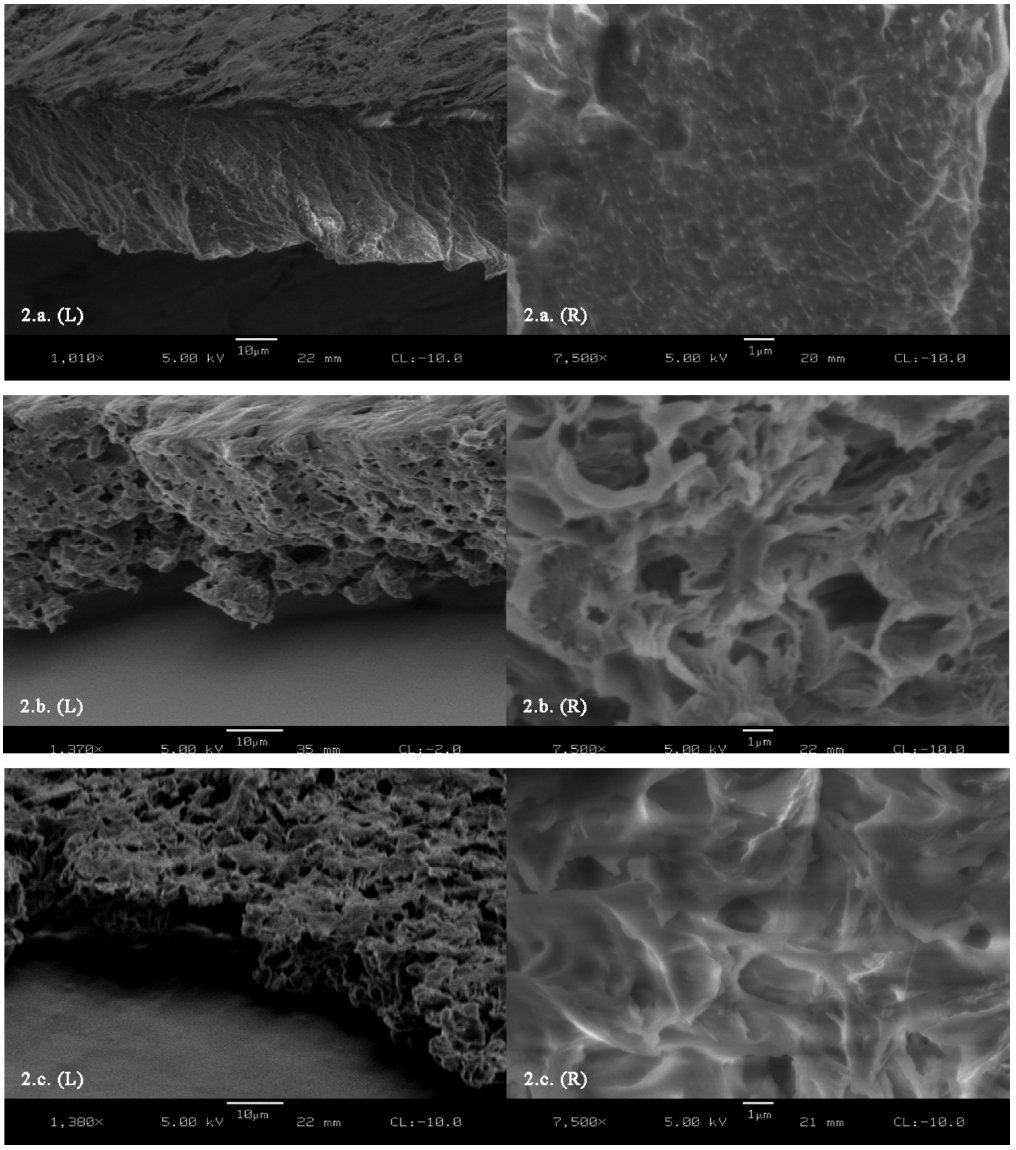

Figure 2. SEM cross-sectional views at different magnifications of PCL films for (a) CALB externally added to films, after 2 h incubation, resulting in 43% film weight loss; (b) CALB embedded within films that have 6.5% CALB initial loading, after 2 h incubation, resulting in 30% film weight loss; (c) CALB embedded within films that have 1.6% CALB initial loading, after 48 h incubation, resulting in 44% film weight loss. L and R designate lower and higher magnification views, respectively.

at film surfaces. Without access to PCL throughout films, externally added CALB does not affect the bulk molecular weight and, therefore, M_n and PDI do not change. Indeed, from review of the literature we conclude that *embedded enzymatic degradation* occurs by a unique mechanism distinguished by a distinctive progression of events that occur during the degradation process. This is further illustrated by comparison of *embedded enzymatic degradation* to bulk chemical hydrolysis of PLA films incubated in buffer. As PLA film hydrolysis proceeds, PLA molecular weight average decreases and PDI moves toward a poisson distribution due to random events of cleavage that occur throughout films.[12]

Cross-sectional images recorded by SEM of partially degraded embedded CALB−PCL films, and PCL films to which CALB was added externally, are displayed in Figure 2. SEM images in Figure 2a recorded for externally added CALB incubation with PCL to 43% film weight loss show a roughened surface with no evidence of degradation below the surface region. This is consistent with the model that, when CALB is added externally, CALB can only access PCL at film surfaces. SEM photographs displayed in Figures 2b,c show cross-sectional views of films with initial embedded CALB loadings of 6.5 and 1.6% that reached weight loss values of 30 and 44%, respectively. In contrast to externally added CALB, incubations of embedded CALB films results in pore formation throughout the material (see cross-sectional images after 70% weight loss in Supporting Information Figure s4). This is explained by porosity; as pores increase in size, they conjoin, creating networks. Furthermore, water continues to reach new film regions where CALB aggregates reside. Once CALB aggregates obtain sufficient water supply they begin actively hydrolyzing adjoining PCL surfaces.

DSC measurements showed significant differences in PCL crystallinity for residual PCL films that had undergone *embedded enzymatic degradation* versus PCL films whose degradation was a consequence of external enzyme addition to media. For example, the 6.5% CALB−PCL embedded system, which underwent a weight loss of 80%, gave a

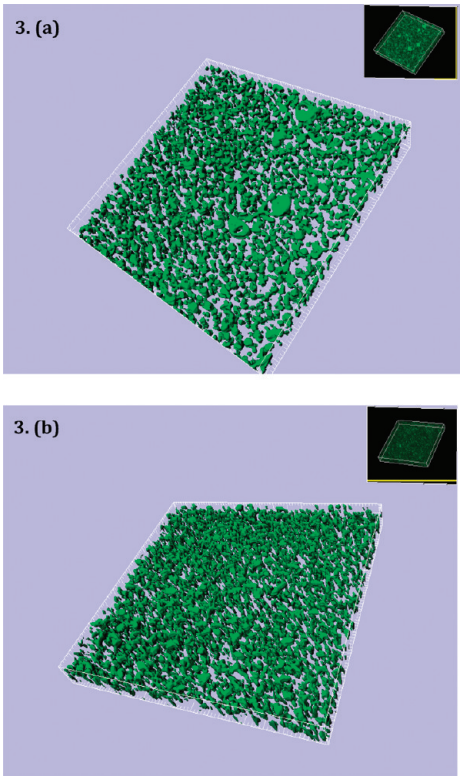

Figure 3. Laser scanning confocal microscopy images: (a) distribution of FITC-tagged CALB in 6.5% CALB-embedded PCL; (b) distribution of FITC-tagged CALB in 1.6% CALB-embedded PCL.

residual film that was 90% crystalline. Furthermore, the 1.6% CALB−PCL system that underwent a weight loss of 80% gave residual films that were 80% crystalline. In contrast, when CALB was added externally and film weight loss reached 80%, film crystallinity was 50%, which is close in value to % crystallinity of untreated films (~50%). Furthermore, analysis by DSC of control films retrieved after incubation without enzyme at 37 °C over 17 days showed no change in film crystallinity. Thus, preferential degradation of PCL amorphous regions by *embedded enzymatic degradation* results in a highly crystalline porous matrix. When CALB is added externally, CALB's access is limited to the films surface, leaving the bulk material unaffected by the enzyme. Consequently, based on the same reasoning, both the crystallinity and molecular weight of residual films from external enzyme addition remain largely unchanged from control films untreated by enzyme.

To determine embedded CALB distribution and the average size of CALB-AOT aggregates in PCL matrices, CALB was labeled with fluorescein isothiocyanate (FITC), and the resulting FITC-CALB embedded PCL films were analyzed by laser scanning confocal microscopy. Resulting images of nonincubated films with 6.5 and 1.6% embedded FITC-labeled CALB are displayed in parts a and b of Figure 3, respectively. The entire film volume was scanned to determine average aggregate size and standard deviation, σ. Values were 6.5 ± 2.4 and 4.0 ± 1.6 μm for PCL−CALB films with 6.5 and 1.6% embedded CALB, respectively. Even though these values are not significantly different, they indicate that by decreasing the concentration of CALB embedded in films there is a corresponding decrease in aggregate size.

Conclusions, Significance, and Future Perspectives. Results above demonstrate that, by surfactant pairing CALB and dispersing CALB throughout PCL films, a novel system resulted that allows wide variations in PCL lifetime. Thus, without copolymerization, changing initial PCL molecular weight or film crystallinity, but by embedding an enzyme within the PCL matrix that is active for PCL hydrolysis, biomaterials were created that degrade by a novel mechanism due to formation of multiple enzyme−surface interfaces throughout films. It is anticipated that the nature of these interfaces can be specifically manipulated by using different hydrolytic enzymes and surfactants and by altering the size and other characteristics of molecular dispersed embedded enzymes. By changing these parameters, we anticipate that it will be possible to further tune bioresorption rates of enzyme-embedded biomaterials without changing the concentration of enzyme within films. Furthermore, future identification of different material−hydrolytic enzyme systems will further expand the impact of *embedded enzymatic degradation* on the field of biomaterials science and engineering.

Acknowledgment. The authors thank the National Science Foundation Industry/University Cooperative Research Center (NSF-I/UCRC) for Biocatalysis and Bioprocessing of Macromolecules at Polytechnic Institute of NYU for their financial support.

Supporting Information Available: Details of all procedures and experiments (Methods section) along with GPC chromatograms and additional SEM cross-sectional views. This material is available free of charge via the Internet at http://pubs.acs.org.

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