Further Studies on Biodegradation of Aliphatic Poly(Ester-Amides)

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SYNOPSIS

Two types of aliphatic poly(ester-amides) (PEAs) were subjected to microbial degradation in basal mineral salt broth, under the attack of a yeast, *Cryptococcus laurentii*, at 20°C. PEA-I copolymers were made by the anionic ring-opening copolymerization of of ε-caprolactone and ε-caprolactam, whereas PEA-II copolymers were synthesized by a two-step polycondensation reaction of hexanediol-1,6, hexanediamine-1,6, and adipoyl chloride. These copolymers were found to be readily degradable under biotic conditions, based on weight loss, GPC, NMR spectroscopy, and tensile property measurements. Compared to abiotic hydrolysis, biodegradation occurred much faster under milder conditions. Furthermore, NMR spectroscopic analysis proved that the biodegradation of poly(ester-amides) involves the enzymatic hydrolysis of ester groups on the backbones of polymers into acid and hydroxyl groups. No breakdown of amide bonds was observed under the given biotic conditions.

INTRODUCTION

The biodegradation of synthetic polymers is of considerable interest to environmentalists and industrial, as well as academic, researchers. The study of the biodegradation of polymers was initiated to prevent polymers from attack by microorganisms in the environment. However, the more important impetus for the early studies of biodegradable polymers was their ecological value.[1-3] The interest in biodegradable polymers for biomedical applications also greatly increased when Schmitt and Polistina[4] introduced poly(glycolic acid) as the suture material Dexon in 1967.

As an important class of biodegradable polymers, aliphatic polyesters have been widely investigated.[5-11] Among them, two poly(α-hydroxy acid)s, poly(glycolic acid), poly(L-lactic acid), and their copolymers, have been successfully used as bioabsorbable sutures and are potentially useful in other biomedical applications, such as controlled drug release, implants, and bone fixation devices.[7-9] Other types of poly(hydroxy acid)s, derived from β, γ, δ, and ε-hydroxy acids, are also degradable, however, at slower rates than the α-hydroxy acids analogs.[10,11]

Unfortunately, aliphatic polyesters lack certain physical properties that are required for practical applications in areas other than in the biomedical area. To develop biodegradable polymers with better physical properties, aliphatic poly(ester-amides), composed of esters and amides, have been studied for the last 15 years. Polydepsipeptides, which are a class of alternating copolymers of α-amino acids and α-hydroxy acids, were reported to be hydrolyzable and biodegradable.[12-15] Helder et al.[16] studied the *in vitro* degradation of nonalternating glycine/DL-lactic acid copolymers. Barrows and coworkers[17,18] also synthesized a series of alternating polyesteramides by using a two-step polycondensation reaction from glycolic acid, diamine, and diacyl chloride, and investigated the *in vivo* degradation of the copolymers for the purpose of developing new surgical implants. However, few reports on the degradation of polyesteramides, which do not contain α-hydroxy or α-amino acid moieties, and which might be potentially useful for more general pur-

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poses, such as packaging and marine use, are found in the literature. Tokiwa et al.[19,20] showed that polyesteramides, made by amide–ester interchange reactions of nylons and polycaprolactone, were degraded by the enzyme *lipase*. In our previous work,[21] hydrolysis in buffer solutions, and microbial degradation under the attack of fungi, *Aspergillus niger* and *Fusarium moniliforme*, of two series of aliphatic nonalternating poly(ester-amides), were conducted.

In this work, further studies on the microbial degradation of these poly(ester-amides), by the yeast *Cryptococcus laurentii* (*Cr. laurentii*), will be presented, and the mechanisms of hydrolysis and biodegradation will be compared.

EXPERIMENTAL

Synthesis

Two series of nonalternating poly(ester-amides), PEA-I and PEA-II, were prepared for biodegradation studies. PEA-I copolymers were made by the anionic ring-opening copolymerization of ε-caprolactone and ε-caprolactam, catalyzed with sodium caprolactam.[22] PEA-II copolymers were synthesized by a two-step polycondensation reaction of hexanediol-1,6, hexanediamine-1,6, and adipoyl chloride, as described previously.[21] The structures of these polymers are presented in Figure 1.

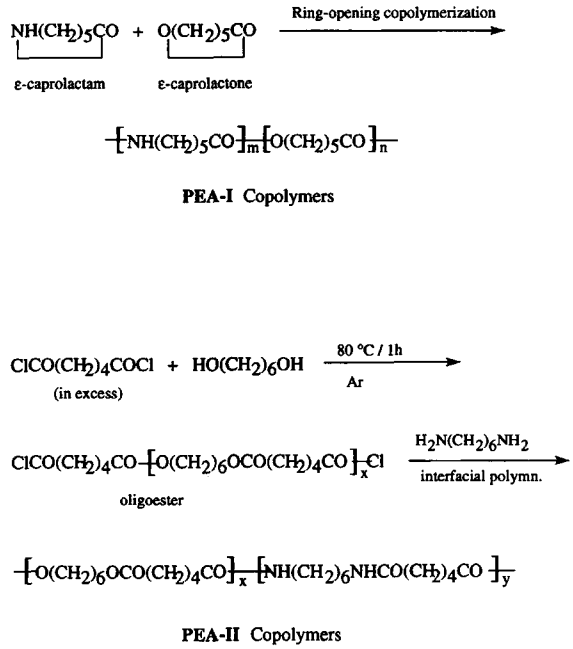


Figure 1 Schematic synthesis of poly(ester-amides).

Techniques

RA40, a PEA-II copolymer, was melt-spun into fibers at 260°C, with a diameter of about 0.1 mm. RA40 film (0.2 mm thick) was molded at 250°C for 2 min, followed by cooling with water. Tensile tests were performed on an Instron (Model 1101) at a speed of 1 in./min.

Intrinsic viscosities of polymers were measured in 90% formic acid solution (0.5 g/dL) at 25.0 ± 0.1°C. NMR spectra of the polymers were obtained on an IBM AF-270 NMR Spectrometer (270 MHz), where CF_3COOD was used as a solvent for all the samples. Molecular weights and molecular weight distributions (MWD) were measured by using a Waters 150-C ALC/GPC, equipped with μ-Styragel HT columns of a 10^4 and two 10^3 Å pore sizes, at a flow rate of 1 mL/min. As solvents, *m*-Cresol and dimethylacetamide (DMAC) were used at 100 and 70°C, respectively. Narrow molecular weight polystyrene standards were used for calibration. The universal calibration method was applied, without using the Mark–Houwink constants *k* and *a*.

Degradation Tests

Materials and Organism

Solution A. K_2HPO_4, 0.7 g, and KH_2PO_4, 0.7 g, were dissolved in 500 mL of distilled water.

Solution B. $M_gSO_4 \cdot 7\,H_2O$, 0.7 g; NH_4Cl, 1.0 g; and $NaNO_3$, 1.0 g, were dissolved in 500 mL of water.

Basal Mineral Salt (BMS) Solution. This solution, used for liquid media tests, was made by mixing the sterilized solutions A and B (500 mL each), followed by adding 1.0 mL of a sterile trace salt solution, containing (per mL of water): NaCl, 5 mg; $FeSO_4 \cdot 7\,H_2O$, 2 mg; $ZnSO_4 \cdot 7\,H_2O$, 7 mg.[24]

Organism. A yeast, *Cryptococcus laurentii* (*Cr. laurentii*), was used to assay the microbial degradation of polymers.

A buffer solution, of pH 7.4, was prepared by dissolving 0.1 M Na_2HPO_4/NaH_2PO_4 in water. Buffer solutions, of pH 4.4 and 10.5, were purchased from Fisher Scientific Co.

Biodegradation Test

Cast Film Test. To expose the maximum surface of the copolymer to the attack of the microorganism, the polymers were cast into thin films (about 30 μm

in thickness). All glassware was thoroughly washed and sterilized. Forty mg of copolymer was placed in a 25 mL culture tube and was dissolved with 8 mL of methanol. The solvent was then evaporated by rotating the tube under a fume hood for 4 days. The tube was in a tilted position during rotation in order to ensure that a large surface of the film would be formed. Ten mL of sterilized BMS broth was then added, and the solution was innoculated with 0.1 mL spore suspension of *Cr. laurentii.* After inoculation, the test tube was kept at 20°C for incubation while rotating. Nylon 6 thin film, cast from formic acid, was also incubated under the same biotic conditions.

After a period of time, the test tubes were taken out from the incubation room. The solvent (water) was removed by freeze-drying in vacuum for two days. Then the solid was extracted by 15 mL methanol three times. The insoluble residue (solid-A) was separated from the solution by filtration. The solution was dried in vacuum for two days and a white solid (solid-B) was obtained. Then, solid-B was weighed and was analyzed by using GPC and NMR spectroscopy.

Fiber Test. RA40 molded film (0.2 mm) and fiber samples were also tested in BMS broth, inoculated with a spore suspension of *Cr. laurentii.* After the incubation, the films or fibers were washed thoroughly with water and were dried in vacuum for two days. These samples were weighed and analyzed by tensile testing and GPC analysis.

Hydrolysis

A piece of polymer film (or fiber) was placed in a vial, containing 10 mL of a buffer solution, with 0.3% sodium azide to inhibit bacterial growth.[16] The vial was maintained at a certain temperature, and the buffer solution was refreshed once the pH change (due to the acidic products on prolonged hydrolysis) was larger than 1.0.

RESULTS AND DISCUSSION

Properties of Copolymers

As shown in Figure 1, PEA-I copolymers were prepared by copolymerization from ε-caprolactone (ester) and ε-caprolactam (amide). PEA-II copolymers were synthesized by polycondensation of adipoyl chloride with hexanediol-1,6 (ester) and hexanediamine-1,6 (amide). We reported previously[21] that both PEA-I and II copolymers showed IR absorptions of both ester and amide groups. It has been proven that these copolymers possess random microstructures, with chains composed of amide and ester units,[21-23,25] (Fig. 1). The compositions, intrinsic viscosities, and DSC data of some PEA-I and II copolymers are shown in Table 1. More detailed characterizations of these copolymers have been reported previously.[21]

Biodegradation of Poly(ester-amides)

Testing of Cast Films. As shown in our previous report,[21] the degradation of the molded film of PEA-I copolymers in agar medium, under the attack of fungus *Fusarium moniliforme,* started from the polymer surface. Both GPC and IR measurements showed that, for polymer films with a thickness of 0.7 mm, only the surface region was degraded in the initial stage, while the bulk of the sample remained the same. Therefore, a liquid medium biodegradation test was designed to assess the degradation of PEA-I copolymer thin films (approx. 30 μm). As shown in Figure 2, the copolymer was first dissolved in methanol and a thin film of the polymer was formed

Table I Composition, Intrinsic Viscosities, and DSC Data of PEA Copolymers[21]

	PEA-I			PEA-II	
Sample[a]	AE45	AE55	AE70	RA40	RA25
Amide Content (mol %)					
Feed	45	55	70	60	75
Found by NMR[b]	31.8	44.3	56.7	54	58
$[\eta]$ (dL/g)	0.26	0.35	0.34	1.30	1.09
T_m (DSC) (°C)	46.7	89.0	115.7	252.2	255.7
ΔH (J/g)	27.3	24.9	—	15.64	41.34

[a] AE70 represents a lactam/lactone copolymer with 70% lactam feed ratio; RA40 is for a random copolymer with 40% diol feed ratio.
[b] Amide % was calculated from the peak integration ratios of methylene groups next to the —COO— and —CONH— groups.

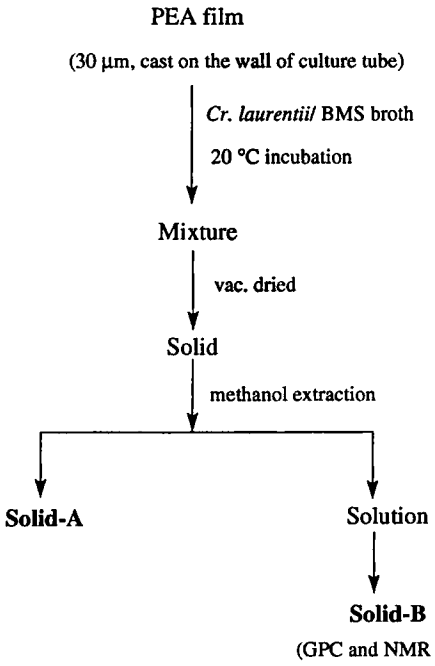


Figure 2 Work-up of microbial degradation in liquid medium.

on the wall of a test tube by evaporating the solvent at room temperature for 4 days. BMS broth was added and then a yeast culture, *Cr. laurentii,* which has proven to be an effective fungus in the degradation of aliphatic polyesters,[26] was inoculated as the microorganism. Under attack of the yeast, the polymer films were broken into tiny pieces after 3 days of incubation at 20°C. As a result of further degradation, the polymer films disappeared in about a week. For the abiotic control samples, the polymer films still remained suspended in the medium even after 20 days, although some broke apart due to mechanical rotation.

After incubation, the reaction mixture was freeze-dried and the residue obtained was extracted with methanol. The insoluble portion, that is, Solid-A, which contains yeast culture, inorganic salts, and inorganic metabolic products, was filtered and discarded. The solution contained undegraded polymer and degraded products (i.e., oligomers). Solid-B was obtained by removing the methanol in vacuum from the solution. The fact that the weight of solid-B decreased with increasing incubation time, as shown in Table II, indicates that the low molecular weight oligomers could have been consumed by the microorganisms as the only carbon source during the incubation. The GPC data for solid-B of AE70, as seen in Table II, showed that the molecular weight of the copolymer was greatly reduced after incubation with the yeast culture at 20°C. In contrast, the control samples showed no decrease in weight, as well as in molecular weight, under the same conditions. In addition, no change in the molecular weight of nylon 6 was observed after 40 days under attack by *Cr. laurentii,* as shown in Table III. This indicates that nylon 6 is not degradable under the given conditions.

Testing of RA40 Fiber. RA40 fiber was also treated with *Cr. laurentii* for a month, and was compared with a control test in abiotic BMS broth. The growth of culture on the fibers was observed after incubation for 2 days. As seen in Figure 3, the tensile properties of this fiber, both tensile strength and elongation, decayed dramatically after the biodegradation. For control samples, no change of the tensile strength was observed after a month, while the elongation was reduced as time increased. On the other hand, some extent of culture growth on the fibers was noted after about a week of incubation in the control test tubes, even though they had been sterilized prior to the test. This phenomenon indicates that the control samples were not in an abiotic condition throughout the experiment. Thus, to some extent, the reduction of the elongation of the fiber can be ascribed to the latter. It is noteworthy that both the tensile strength and elongation of this fiber, as shown in Figure 3, remained unchanged after 40 days of treatment in pH 7.4 buffer solution at 22°C, which can be regarded as a fully abiotic treatment, because 0.03% of NaN_3 was added to the buffer solution to, i.e., non-abiotic conditions inhibit the growth of microorganisms.[16] Similarly, 0.2 mm thick films of RA40 were tested in BMS broth under attack by *Cr.*

Table II Weight Recovery and GPC Data of AE70 Before and After Biodegradation

Time (Days)	Sample No.	Wt (%)	M_w	M_n	M_w/M_n
0	1	97.2	21,875	15,775	1.387
	2	96.6	27,962	20,328	1.376
4	1	95.8	17,273	4,027	4.290
	2	94.6	18,507	4,878	3.794
8	1	72.4	12,048	2,196	5.487
	2	76.8	10,942	2,071	5.284
14	1	52.9	8,070	1,881	4.290
	2	57.7	8,248	1,803	4.575
19	1	58.2	6,499	1,658	3.919
	2	51.5	7,528	1,739	4.328
	3	56.9	4,247	1,439	2.952
19	control-1[a]	96.5	26,985	19,825	1.361
	control-2	97.0	27,867	20,483	1.360

[a] Abiotic conditions.

Table III GPC Data of Nylon 6 Treated with *Cr. laurentii* (in *m*-cresol)

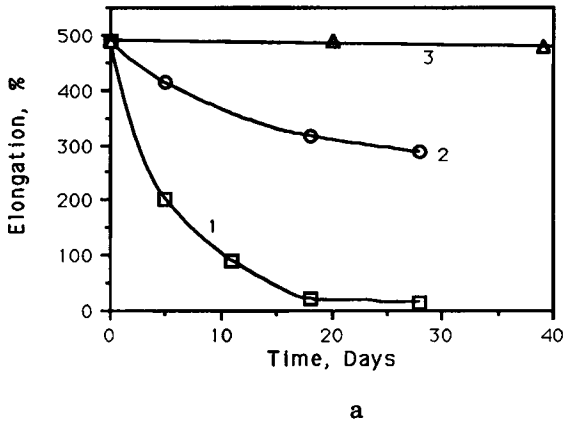
	Original		15 Days		40 Days	
Item	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
M_w	157,570	123,871	141,439	139,247	148,183	148,811
M_nw	61,602	53,012	63,833	63,885	66,157	65,896
M_w/M_n	2.56	2.34	2.22	2.18	2.23	2.26

laurentii and were compared with control samples. As seen in Figure 4, significant weight loss was observed for the sample treated under biotic conditions, while the weight loss of the control samples was negligible.

Mechanism of Biodegradation

Based on the above results, it can be concluded that significant microbial degradation of poly(ester-amides) occurred in a short time period, under the attack of *Cr. laurentii,* whereas no appreciable degradation was observed under corresponding abiotic conditions for a considerable period. As discovered by FTIR spectroscopy in our previous work,[21] the ester concentration of the poly(ester-amides), exposed to *F. moniliforme,* was shown to decrease. However, in all cases, the amide groups remained virtually unchanged, for there were no significant decreases in the peak heights of both the amide-I and II bands.

In this work, ^{1}H–NMR spectroscopy was applied to assess quantitatively the changes in the relative concentrations of ester, amide, and hydroxyl groups, by determining the peak integration of the methylene group next to these groups. As noted in Figure 5, δ 4.0–4.2 represents the absorption of methylene hydrogens in $CH_2O—CO—$ (ester), δ 3.3–3.7 account for those in $CH_2—NH—$ (amide), and δ 4.2–4.4 for $CH_2—OH$ (hydroxyl end group), respectively. Thus, the relative concentration of ester, amide, and hydroxyl groups of the samples can be calculated, based on the peak integration of the NMR spectra. As noted in Figure 6, the ester concentration decreased linearly with the incubation time, whereas the hydroxyl group concentration in-



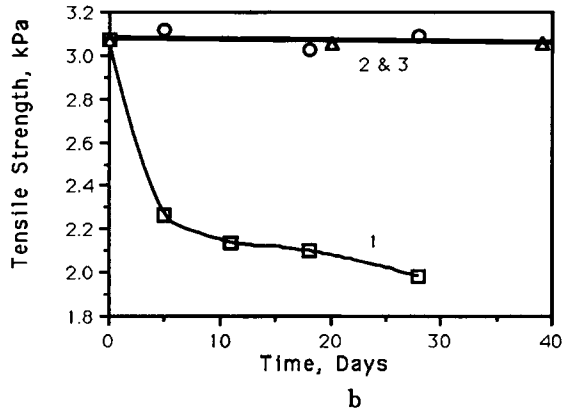


Figure 3 Tensile properties of RA40 fiber after biodegradation and hydrolysis. (1) Yeast culture, 20°C; (2) Control, 20°C; (3) pH 7.4 buffer (with 0.03% NaN_3), 22°C.

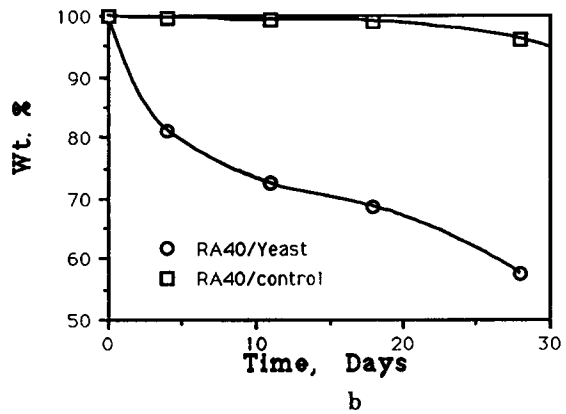


Figure 4 The weight remaining of RA40 film (0.2 mm thick) after degradation by *Cr. laurentii.*

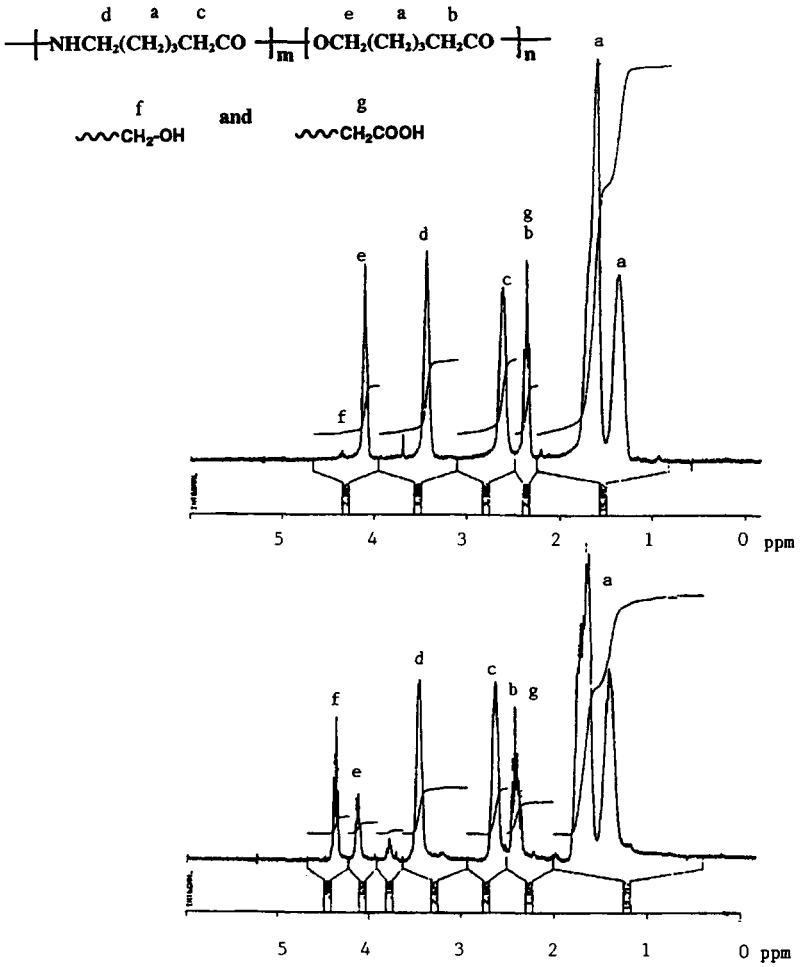


Figure 5 NMR spectra of AE70 vs. degradation by *Cr. laurentii.* (Upper) original sample; (Lower) after 6 days of incubation.

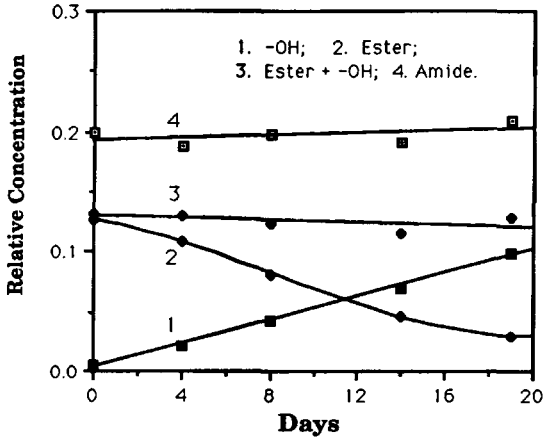


Figure 6 Hydroxyl, ester, and amide concentrations of AE70 vs. biodegradation by *Cr. laurentii.* Relative concentration = I_i/I_a, where I_a is the integration of peak *a*, which represents middle methylene protons of both amide and ester units). I_i represents integration of peak *e* (ester), *d* (amide), or *f* (hydroxyl).

creased linearly. The concentration of the amide component, as well as the total concentration of ester and hydroxyl groups, remained constant.

Thus, the results of the NMR spectroscopic indicate that the biodegradation of poly(ester-amides) takes place, as expected, primarily via the hydrolysis of the ester group into acid and hydroxyl groups, followed by the utilization of the low molecular weight products by microorganisms.

Hydrolysis

This work considers biodegradation as a process of molecular degradation, enhanced by enzyme, as compared to abiotic hydrolysis, where only simple hydrolytical degradation is involved.[5,27] As mentioned above, both PEA-I and II copolymers underwent microbial degradation, while little or no changes were observed for the corresponding control samples. This indicates that simple hydrolysis would

not occur under these conditions. However, at higher temperatures, these copolymers are also hydrolytically degradable, as we reported previously.[21,28] Based on the results of weight loss and GPC analysis, the hydrolysis of these copolymers was greatly enhanced by increasing the temperature from 37°C to 55°C. It was also observed that the degradation of the copolymers was faster in both acidic (pH 4.4) and basic (pH 10.5) media than it was in a neutral medium (pH 7.4).[21,28] This suggests that the hydrolytic degradation of poly(ester-amides) are catalyzed by both H^+ and OH^- ions.[16]

Comparison of Microbial Degradation and Hydrolysis

The hydrolysis of both types of poly(ester-amides) in buffer solutions needs relatively harsh conditions (e.g., 37°C or higher) and a longer time. However, the degradation of these copolymers by microorganisms can occur under mild conditions (e.g., room temperature), in a shorter time period. Both microbial degradation and hydrolysis of the poly(ester-amides) involve the breakdown of the ester bonds.[16] For hydrolysis, the reaction is catalyzed by H^+ or OH^- ions.[16] For biodegradation, however, in addition to the hydrolysis of ester bonds, which is catalyzed by the generated enzymes, other metabolic reactions are also possible, including the consumption of the oligomers or small molecule products by the microorganisms.[6]

CONCLUSIONS

Both types of poly(ester-amides), PEA-I and PEA-II, are readily degradable under the attack of fungus *Cr. laurentii,* based on weight loss, GPC, NMR, and tensile property measurements. The biodegradation of these copolymers occurred via surface erosion, catalyzed by enzymes, while abiotic hydrolysis occurred not only on the surface, but also in the bulk of the copolymers.[21,28] Compared to hydrolysis, biodegradation was found to be much faster. Furthermore, NMR spectroscopic analysis proved that the biodegradation of poly(ester-amides) involves the enzymatically catalyzed hydrolysis of ester groups on the backbones into acid and hydroxyl groups, followed by the utilization of degraded products by the microorganisms. No breakdown of amide bonds was observed under the given experimental conditions.

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