

Article

New Biocompatible Polyesters Derived from α -Amino Acids: Hydrolytic Degradation Behavior

Naomi Cohen-Arazi *, Abraham J. Domb and Jeoshua Katzhendler

Institute of Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Israel; E-Mails: avid@ekmd.huji.ac.il (A.J.D.); katzhe@cc.huji.ac.il (J.K.)

* Author to whom correspondence should be addressed; E-Mail: naomic@ekmd.huji.ac.il; Tel.: 972-2-6758691; Fax: 972-2-6757076.

Received: 20 September 2010; in revised form: 2 October 2010 / Accepted: 8 October 2010 / Published: 13 October 2010

Abstract: New polymers were synthesized from α -hydroxy acids derived from the natural amino acids Ile, Leu, Phe, and Val, combined with lactic acid, glycolic acid and 6-hydroxyhexanoic acid by direct condensation. The toxicity was determined and the degradation process of these polyesters was investigated under physiological conditions by analyzing the composition of the degraded polymers and the oligomers cleaved in the buffer medium. The polymers were found to be non toxic to two cell lines. Polymers displayed a biphasic degradation behavior. In most cases, a linear relationship was found between the weight loss constant and the hydrophobicity of the polymers, Log P. Regarding the second stage of weight loss, it is apparent that polymers derived from α -hydroxy(L)isoleucine ((L)HOIle) and α -hydroxy(L)Valine ((L)HOVal) degraded much faster than those derived from α -hydroxy(L)leucine ((L)HOLEu) and α -hydroxy(L)phenylalanine ((L)HOPhe), probably due to different spatial orientation of the side chains. Copolymers of 6-hydroxyhexanoic acid displayed slow degradation rates as expected, whereas the degradation profile of copolymers of lactic acid was similar to the other homopolymers. These new polyesters may serve as potential biocompatible materials for medical applications.

Keywords: α -hydroxy acids; polyesters; toxicity; degradation; drug delivery

1. Introduction

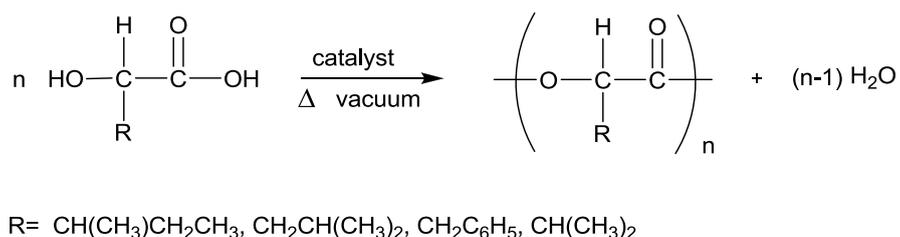
The biodegradable polymers that have been most intensively investigated are aliphatic polyesters derived from the following monomers: lactide, glycolide, butyrolactone and caprolactone [1,2]. Their polyesters and copolyesters represent the main group of interest [3–9] due to their long history of safety. Interestingly, little has been reported on others hydroxy acid based polyesters, probably due to the limited availability of other hydroxy acids from natural sources or complication in their synthesis [10]. Biodegradable polymers that have one or more stereogenic center(s) in the repeat unit (namely, optically active polymers), offer advantageous features when used as carriers for drug delivery [11]. In light of the above, our laboratory has reported expansion of the biodegradable polymers range to new poly(hydroxy acids) carrying various stereogenic centers. Viewing the scope of work in the area reveals that the selection of available chiral hydroxy acids is limited and most of the work been done either with lactic acid or derivatives of acrylic acid attached to chiral moiety. The monomers of the homopolymers prepared in our laboratory were synthesized from natural α -amino acids using a straightforward reliable and inexpensive method of diazotization of α -amino acids (Scheme 1) [12,13].

Scheme 1. Synthesis of the α -hydroxy acids.



Linear homopolyesters of (S)-2-hydroxy-4-methylpentanoic acid ((L)HO-Leu), (S)-2-hydroxy-3-phenylpropionic acid ((L)HO-Phe), (S)-2-hydroxy-3-methylpentanoic acid ((L)HO-Ile), (S)-2-hydroxy-3-methylbutanoic acid ((L)HO-Val) and their copolyesters, either with one of the above hydroxy acids (1:1) or with (L)lactic acid (1:1), glycolic acid (1:1) or 6-hydroxyhexanoic acid (1:1), were prepared by direct condensation in bulk (Schemes 2 and 3) and checked for toxicity and degradation.

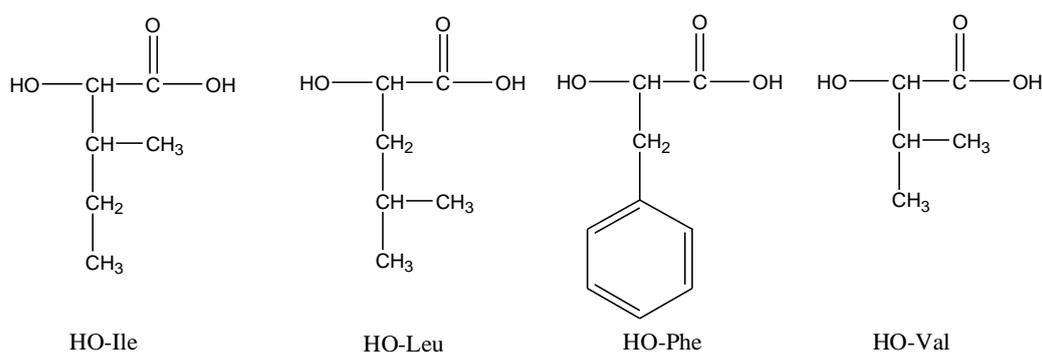
Scheme 2. Direct condensation of the α -hydroxy acids.



During the degradation process, high-molecular-weight, water-insoluble macromolecules are converted to small, water-soluble molecules by a hydrolytic cleavage of labile bonds in the polymer backbone. The polymers undergoing this type of degradation include aliphatic polyesters, polyamides, poly(cyanoacrylates), polyanhydrides, polyacetals, and poly(ortho esters). Among these polymers,

PLA, PGA, and PLGA are well known. The hydrolytic degradation of poly(α -hydroxyacids) is mainly influenced by four major factors: (1) the hydrolysis rate constant of the ester bond; (2) the diffusion coefficient of water in the polymer matrix; (3) the diffusion coefficient of the chain fragments within the polymeric matrix and (4) the solubility of the degradation product. The degradation behavior of polyesters has been intensively investigated, as well as the mechanism of their degradation [14]. Several theories have been developed to describe the degradation of aliphatic polyesters [15]. Most of the models follow first or pseudo first order kinetics and the disadvantage of these models is that the autocatalytic behavior of the degradation is not incorporated. Lately new models were developed based on the kinetics of the autocatalytic hydrolysis of aliphatic polyesters which can be used for homo and copolyesters [14,15].

Scheme 3. α -hydroxy acids synthesized by diazotization of amino acids.



Herein, we present the toxicity and degradation profile of the newly prepared polyesters. The study is focused on two main elements: composition of the polymer matrix during the hydrolysis (weight loss and molecular weight decrease) and composition of the degradation products.

2. Results and Discussion

2.1. Synthesis of Polymers

The molecular weights of the polymers containing only HOIle, HOLeu, HOPhe and HOVal (Table 1, entries 2 to 11) are around 2000 Da. The introduction of lactic acid or glycolic acid to the hydrophobic polymers (Table 1, entries 12 to 20) slightly increases the molecular weight of the polymer. However, copolymers with hydroxyhexanoic acid (Table 1, entries 21 to 24) reached a significantly higher molecular weight of more than 5000 Da, due to its long aliphatic chain. In general, the polymerization of hydrophobic α -hydroxy acids by the polycondensation method results in slightly lower molecular weights (in the range of 2000–3000 Da) than that of lactic acid or glycolic acid [16].

Copolymers with glycolic acid and hydroxyhexanoic acid reveal a lower optical activity than homopolymers and copolymers with lactic acid (LA), since these two monomers lack a chiral center. The copolymers with hydroxyhexanoic acid demonstrate the lowest optical activity, probably because the long aliphatic chain of caproic acid attenuates the cooperative rotational strength of the chiral centers along the polymer.

The extent of the polymer's solubility in chloroform is higher than that of PLA. They are also soluble in THF and ACN, but not in water.

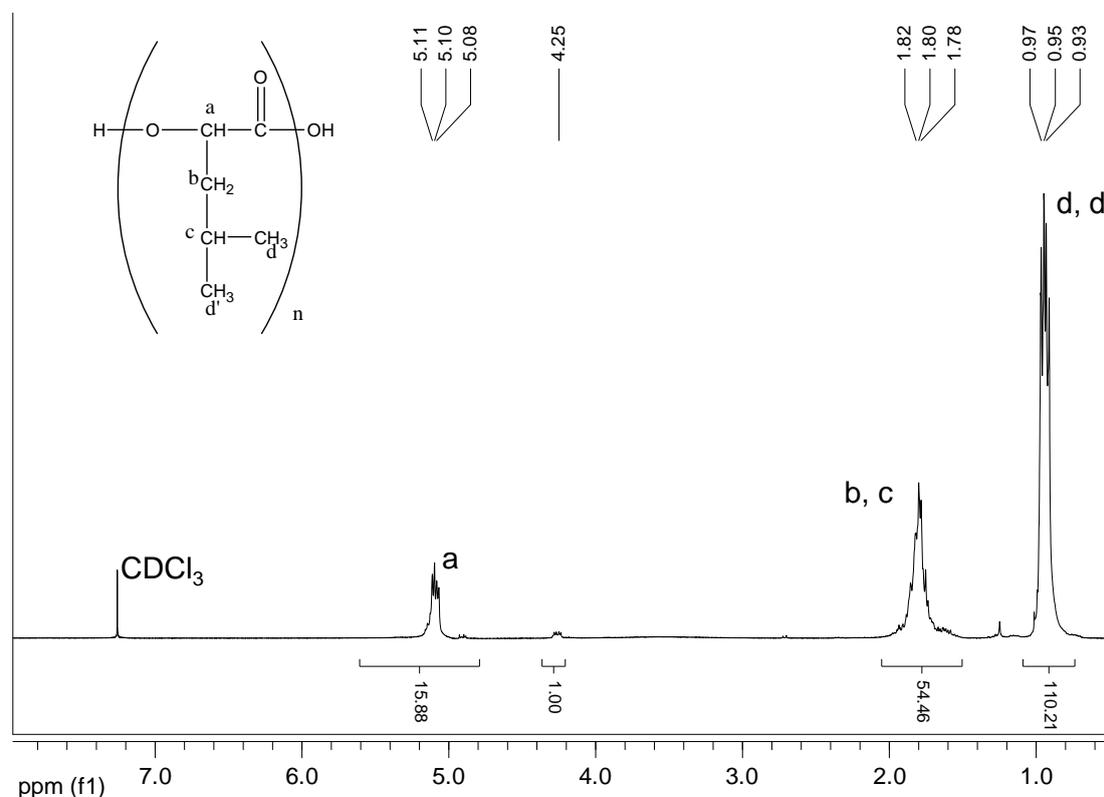
Table 1. Characterization of the prepared polymers.

	Polymer	Mw ^a (Da)	Mn ^a (Da)	Optical activity [α] _D ^b (lit. value)	Solubility (g/L) ^c			Tg(°C) ^d (Lit value)	Δ H (J/gr) ^d	Water contact angle (θ) ^e
					CHCl ₃	THF	ACN			
1	LPLA	2800	2600	-139 (-141) [17]	250	18	<2%	49.0 (49–60) [17]	-6.3	37.1
2	Poly(L)HOIle	1000	800	-10	>600	150	60	-10.7	-4.1	53.0
3	Poly(L)HOLeu	2300	1900	-43	>600	80	120	-8.8	-3.7	76.2
4	Poly(L)HOPhe	2500	2200	-37	>600	37	47	32.0* (38–50) [18]	-5.9	85.8
5	Poly(L)HOVal	1000	700	-32	>600	122	65	16.8	-4.3	73.7
6	Poly(L)HOIle- HOLeu	1700	1400	-37	360	450	400	-36.7	-2.2	89.7
7	Poly(L)HOIle- HOPhe	1900	1400	-22	60	500	330	-42.0	-2	86.3
8	Poly(L)HOIle- HOVal	800	800	-12	>600	520	280	-45.1	-3.7	37.0
9	Poly(L)HOLeu- HOPhe	2500	1900	-31	110	400	400	-36.2	-2.3	89.8
10	Poly(L)HOLeu- HOVal	2500	1800	-42	360	500	400	-45.0	-2.2	107.5
11	Poly(L)HOPhe- HOVal	1700	1300	-26	150	500	500	-44.1	-2.7	100.5
12	Poly(L)HOIle-LA	1800	1600	-53	>600	32	75	5.4	-3.013	67.4
13	Poly(L)HOLeu-LA	2300	1900	-60	>600	147	59	11.2	-5.31	78.4
14	Poly(L)HOPhe-LA	2500	2300	-55** (-66) [18]	>600	410	41	25.9** (38–50) [18]	-11.8	99.1
15	Poly(L)HOVal-LA	1700	1400	-70	>600	78	33	29.2	-6.8	87.7
16	Poly(L)HOIle-GA	1700	1400	-22	>600	400	300	-41.2	-3.0	54.7
17	Poly(L)HOLeu-GA	2800	2100	-46	>600	400	400	-43.2	-2.8	95.7
18	Poly(L)HOPhe-GA	2400	1700	-22	220	500	360	-36.2	-5.6	82.7
19	Poly(L)HOVal-GA	2200	1600	-29	>600	500	500	-45.9	-3.7	55.1
20	Poly(L)HOIle-CA	2500	2100	-4	540	500	500	-63.9	-7.0	46.1
21	PolyHOLeu-CA	5400	2800	-24	180	500	400	-69.3	-9.7	57.0
22	PolyHOPhe-CA	4100	2200	-13	360	400	340	-46.7	-4.3	73.9
23	PolyHOVal-CA	2800	1800	-5	549	600	360	-73.2	-6.0	50.4

^a The molecular weights were determined by GPC; ^b Specific optical rotation ($c = 1-1.2$, in CHCl₃, at 25 °C); ^c Solubility of the polymers in several organic solvents; ^d Tg and Δ H were determined by DSC at 10 °C/min; ^e Water contact angle was determined using a contact angle goniometer. *The molecular weight of the PolyHOPhe corresponding to the literature value is higher than 3000 Da. **The polymer corresponding to the literature value contains 70% HOPhe unit.

$^1\text{H-NMR}$ spectrum of PolyHOLeu is shown in Figure 1. $^1\text{H-NMR}$ spectroscopy confirmed the polymerization of the monomers by the appearance of a CH peak between 5.1 and 5.3 ppm corresponding to the ester group CH-COO-CH , and the significant disappearance of the CH-OH peak of the hydroxy acid at 4–4.3 ppm. Polymers containing lactic acid unit show a methyl signal around 1.0 ppm, whether polymers containing hydroxyhexanoic acid show several peaks from 1.0 to 2.5 ppm corresponding to the alkyl chain.

Figure 1. $^1\text{H-NMR}$ spectrum of PolyHOLeu.



From $^1\text{H-NMR}$ data, the M_n value of the polymer can be evaluated. The ratio between the integration of the two CH signals (of the backbone at 5.10 ppm, and of the end group at 4.25 ppm) provides the degree of polymerization of 15.88. Since the molecular weight of the monomeric unit is 114.14, the M_n value of the analyzed polymer is found to be 1830 Da (including the H and OH end groups). This calculated data is close to the M_n determined by GPC (1900 Da). By the same method, M_n of all homopolymers were calculated and compared to the M_n obtained from GPC analysis (Table 2).

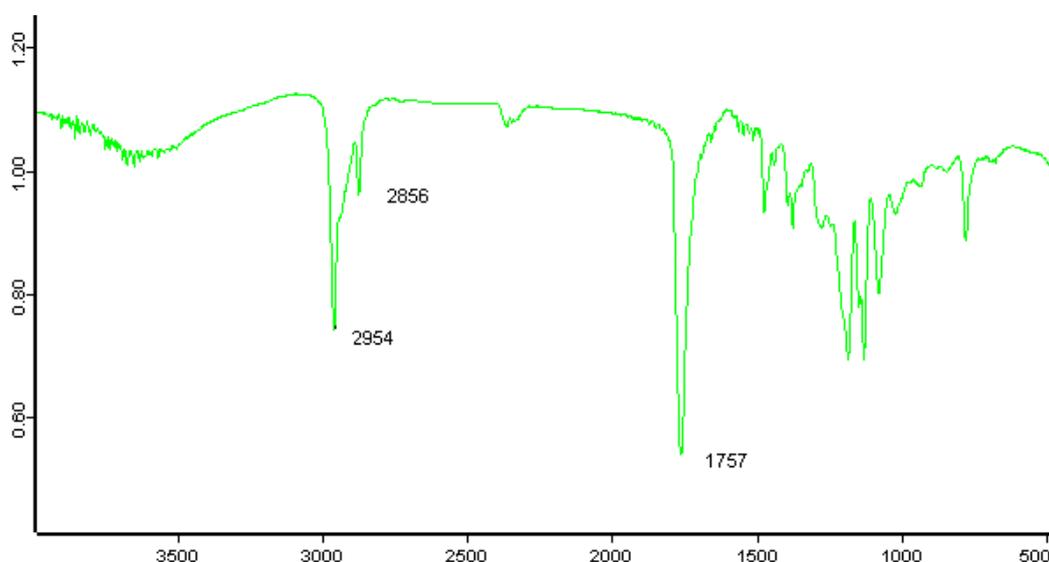
Table 2. Comparison between M_n obtained from GPC and M_n obtained from the $^1\text{H-NMR}$ spectrum for the analyzed polymers.

Polymer	M_n (Da, GPC)	M_n (Da, $^1\text{H-NMR}$)
Poly(L)HOIle	800	710
Poly(L)HOLeu	1900	1830
Poly(L)HOPhe	2200	2100
Poly(L)HOVal	700	650

For all homopolymers, the M_n values calculated from $^1\text{H-NMR}$ and the M_n values obtained from GPC were similar. It seems likely that polystyrene polymers are suitable as standards for GPC determination of molecular weight of the poly α -hydroxyacids. In the case of copolymers, the M_n values could hardly be calculated from $^1\text{H-NMR}$ spectrum due to the overlapping of peaks.

The infrared spectrum of PolyHOleu is depicted in Figure 2. All polymers show a major ester band between 1750 to 1765 cm^{-1} and a small acid band around 1640 cm^{-1} . They also show 2 bands corresponding to alkyl groups/chains, 2850 – 3000 (stretch) and 1350 – 1480 (bending) cm^{-1} . The intensity and broadness of the bands increase with the length of alkyl pendant chains along the polymers; hydroxyhexanoic acid polymers exhibit the largest absorption areas.

Figure 2. IR spectrum of PolyHOleu. The typical ester carbonyl band can be observed at 1757 cm^{-1} . IR spectroscopy was performed on the polymer sample cast on NaCl plate from a chloroform solution.



2.2. Toxicity

To analyze the biocompatibility of the prepared polymers, the various polymers were screened for acute toxic effects using a lactate dehydrogenase (LDH) assay and were found to be not toxic. Thereafter, the biocompatibility of the newly synthesized polymers was evaluated on a neuronal (PC12 pheochromocytoma) cell line following differentiation and an endothelial (bEnd.3) cell line following adhesion and proliferation. Several polymers induced neuronal differentiation, and none of the tested polymers reduced the adhesion of endothelial cells (manuscript in preparation) [19].

2.3. In vitro Hydrolytic Degradation of the Polymers

2.3.1. Weight Loss Analysis

The data for most polymers showed a bimodal behavior of monophasic or biphasic degradation kinetics, where the initial stage is defined by a fast rate (burst stage), while the second stage is characterized by a slow rate. The weight loss data was fitted (by a non linear regression) to either one

(in case of one stage) or two exponential growth terms [20] (in case of two reaction stages) as is depicted in Equation 1, using the WinNonlin program (Pharsight). The two-parameter (P_1 and k_1) model describes mainly the initial part of the degradation very well, whereas the four-parameter model is appropriate for fitting hydrolytic degradation over the entire time period.

$$\%P(\text{degrad}) = P_1 \times (1 - e^{-k_1 t}) + (P_0 - P_1) \times (1 - e^{-k_2 t}) \quad (1)$$

$\%P(\text{degrad})$ is the weight loss percentage of the polymer at time point t . P_1 is the percentage of the weight loss at the end of the first step of degradation. P_0 is the total weight loss of the polymer, which is 100%. k_1 and k_2 are the rate constants of the two steps, respectively. Kinetic parameters for each degraded polymer are summarized in Table 3.

From the table, it is apparent that most polymers degrade according to biphasic kinetics. The first stage of the reaction is fast ($t_{0.5}$ varies in the range of several hours to 40 hours) and its amplitude is 20–30%. The ratio between the rate constants of the two reaction stages (k_1/k_2) is of several folds: 35–349, indicating the difference in the hydrolytic rates of both phases.

It is generally accepted that surface hydrolysis may occur at a different rate than the core hydrolysis due to factors controlling water penetration [21,22]. If hydrolysis is slow compared to diffusion, the complete cross section of a polymer matrix is affected by erosion that has been named bulk erosion or homogenous erosion [23]. With increasing degradation velocity, however, erosion becomes a surface phenomenon because water is consumed mainly on the surface by hydrolysis. This has been designated as surface erosion or heterogeneous erosion. Only fast degrading polymers, such as polyanhydrides and poly(ortho esters), have been reported to be surface eroding. The kinetic factors for the process include the surface concentration of ester bonds and the surface to volume ratio of the sample. For example, the degradation mechanism of crystalline PLA's with a parabolic-type degradation pattern [24] can be explained by the following three processes: (1) dissolution of oligomer, (2) preferential hydrolysis of a relatively low molecular weight copolymer in the amorphous regions leading to a narrow molecular weight distribution of the higher molecular weight and crystalline copolymers, and (3) rate-limiting degradation of the copolymer in the crystalline regions. The resulting degradation of crystalline polymer mainly proceeds by the processes (1) and (2) in the initial stage, followed by (3), giving a parabolic-type degradation pattern.

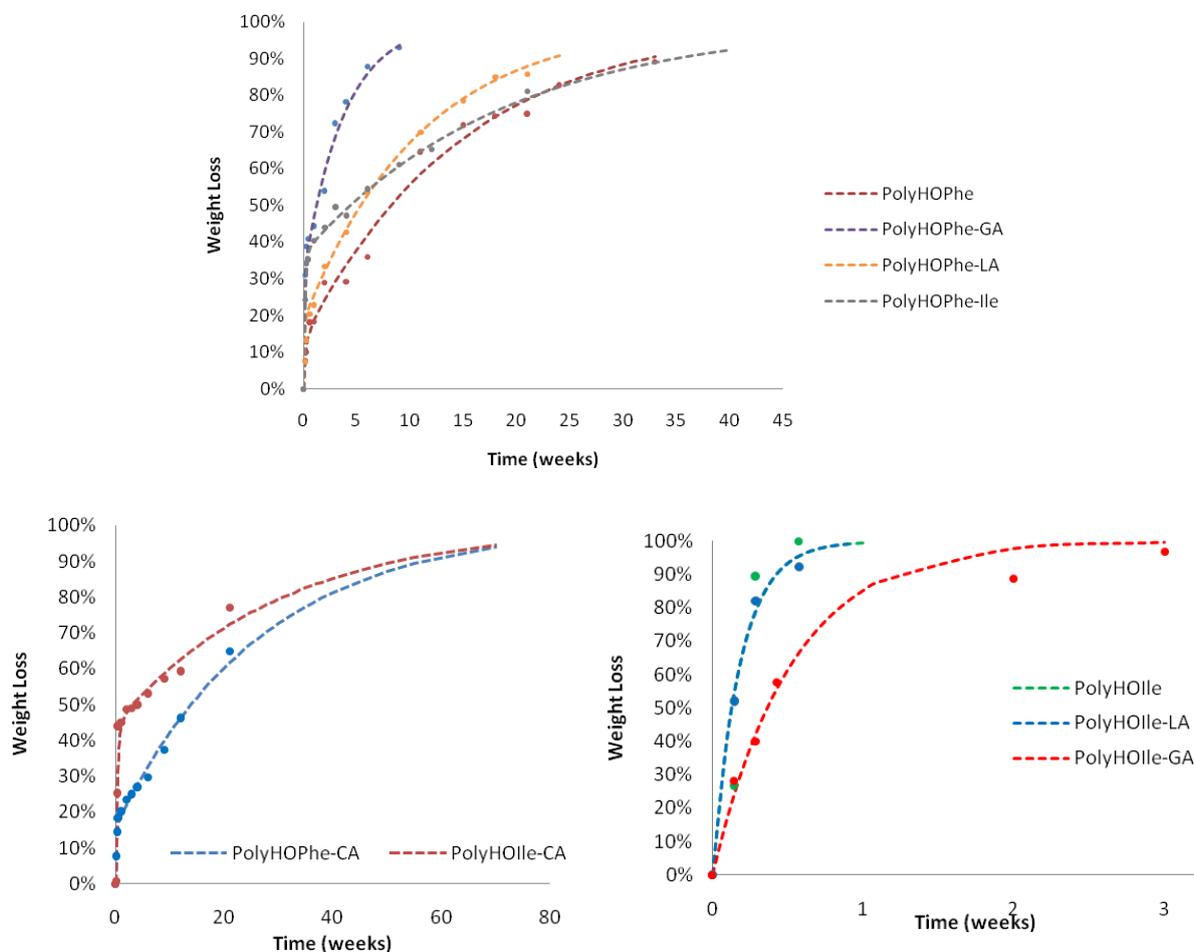
This dissimilarity between the two ongoing processes (cleavage and diffusion) can explain the biphasic reaction in the degradation behavior of the present polymers. At contact with the buffer medium, small polymer chains readily dissolve in the aqueous medium and end groups, which are on the surface of the injected polymer, cleave and dissolve in the medium (burst stage). Following this first contact with the surrounding aqueous medium, the injected polymer is more resistant, and the diffusion of water molecules into the polymer core is the rate limiting factor (second stage).

The biphasic reaction of copolymers of HOPhe and copolymers of HOIle is illustrated in Figure 3. Copolymers of HOPhe with LA and HOIle have a half life of about three months in the burst phase whereas the insertion of GA reduces the half life time to less than a month. Indeed Panyam *et al.* [25] showed that the initial loss rate is found to be more rapid in PLGA polymers with high glycolic acid content. This is consistent with the more hydrophilic nature of the PLGA compared to lactide homo-polymers [25].

Table 3. The calculated rate constants (k_1 , k_2 , P_1 and $t_{(1/2)}$ of each step) resulting from polymers degradation fitting using Equation 1.

Polymer	$k_1(\text{week}^{-1})$ $t_{(0.5)} \text{ h}$	P_1	$k_2(\text{week}^{-1})$ $t_{(0.5)} \text{ weeks}$	k_1/k_2
PolyHOPhe	4.75 24.5	13.45	0.0669 10.4	71
Poly-HOPhe-CA	6.12 19.0	15.95	0.0373 18.6	164
PolyHOPhe-GA	17.50 6.7	31.60	0.265 2.6	66.79
PolyHOPhe-LA	12 9.7	18.83	0.091 7.6	34.83
PolyHOPhe-HOVal	9.87 11.8	47.6	0.093 7.5	107
PolyHOPhe-HOIle	7.10 16.4	37.05	0.053 13.1	134
PolyHOPhe-HOLEu	8 14.6	22.38	0.0737 9.4	276
PolyHOLEu	2.605 44.8	44.4	0.073 9.5	36.3
Poly-HOLEu-CA	3.62 32.2	21.96	0.027 25.6	349
PolyHOLEu-GA	10.52 11.1	31.6	0.291 2.4	46.5
PolyHOLEu-LA	6.46 18.0	23.9	0.0719 9.6	89.8
PolyHOLEu-HOVal	5.06 23.0	33.78	0.028 24.7	316
PolyHOLEu-HOIle	5.08 22.9	26.9	0.0587 11.8	87.6
PolyHOIle	5.42 21.5	–	–	–
PolyHOIle-CA	2.91 40.0	44.62	0.0328 21.1	88.7
PolyHOIle-GA	1.91 60.9	–	–	–
PolyHOIle-LA	5.5 21.2	–	–	–
PolyHOVal	4.48 26.0	–	–	–
PolyHOVal-CA	2.91 40.0	66.47	0.0431 16.1	158
PolyVal-GA	3.97 29.3	–	–	–
PolyHOVal-LA	4.67 24.9	–	–	–
PolyHOVal-HOIle	5.10 22.8	–	–	–

Figure 3. Hydrolysis of copolymers of HOPhe and copolymers of HOIle monitored by weight loss. The broken curves correspond to the theoretical rates whereas the points are the experimental values.



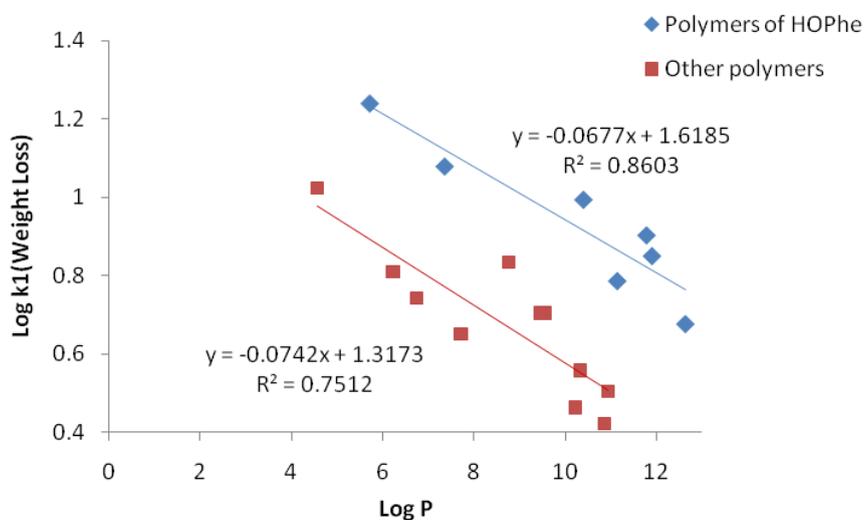
This trend is inverted when evaluating copolymers of HOIle. The homopolymer and the copolymer with LA present a monophasic behavior and completely degrade in less than a week, whereas it takes three weeks for the HOIle-GA copolymer. These modulations are related to many aspects such as morphology of the sample, crystallinity, molecular weight of the polymer, and hydrophilicity, which may have significant impact on the degradation [21,24].

The effect of hydrophobicity on the degradation behavior of the polymers during the burst phase was also studied. Although the water contact angle gives an indication of the hydrophobicity of the polymer, no correlation was found between the water contact angle of the polymers and the weight loss constant, neither with Log P. Many factors are responsible for the contact angle of a polymer's surface including surface contamination, heterogeneity of the surface structure, reorientation, mobility or roughness of the surface segment, swelling, and deformation [26]. The properties of a polymer cast from a hydrophobic solvent (chloroform) on glass are different from the same polymer immersed into an aqueous medium. This might be the reason for the lack of correlation between degradation constants and contact angle results.

However, a linear correlation between the weight loss constant Log k_1 and the hydrophobicity Log P was found when the polymers were divided into two sets. The first set included homo and

copolymers of HOPhe, while the second set involved homo and copolymers of HOVal, HOLeu and HOIle. Log P of the polymers was calculated by the ALOGPS 2.1 program, employing oligomers of 10 units for each polymer. The linear correlations are delineated in Figure 4.

Figure 4. Linear Correlation between Log k_1 and Log P for the two sets of polymers.



It can be concluded that the higher the hydrophobicity of the polymers, the slower the degradation rate. There is no linear dependence between the hydrophobicity (logP) and log k_2 , since the degradation of the inner part of the sample depends not only on the hydrophobicity but also on the packing density of the matrix core.

Three polymers were not included in the linear correlation: PolyHOVal-LA, PolyHOIle-GA and PolyHOVal-GA since they are very hydrophilic and rapidly degrade to completion. The degradation rate of these polymers is not dependent on their hydrophobicity.

The difference between polymers of HOPhe and the other polymers can be explained by the fact that HOPhe is able to intercalate water molecules due to its spatial oriented aromatic ring, so that water molecules are more accessible for degradation during the burst stage. Thus, k_1 of HOPhe copolymers are enhanced compared to other hydrophobic polymers.

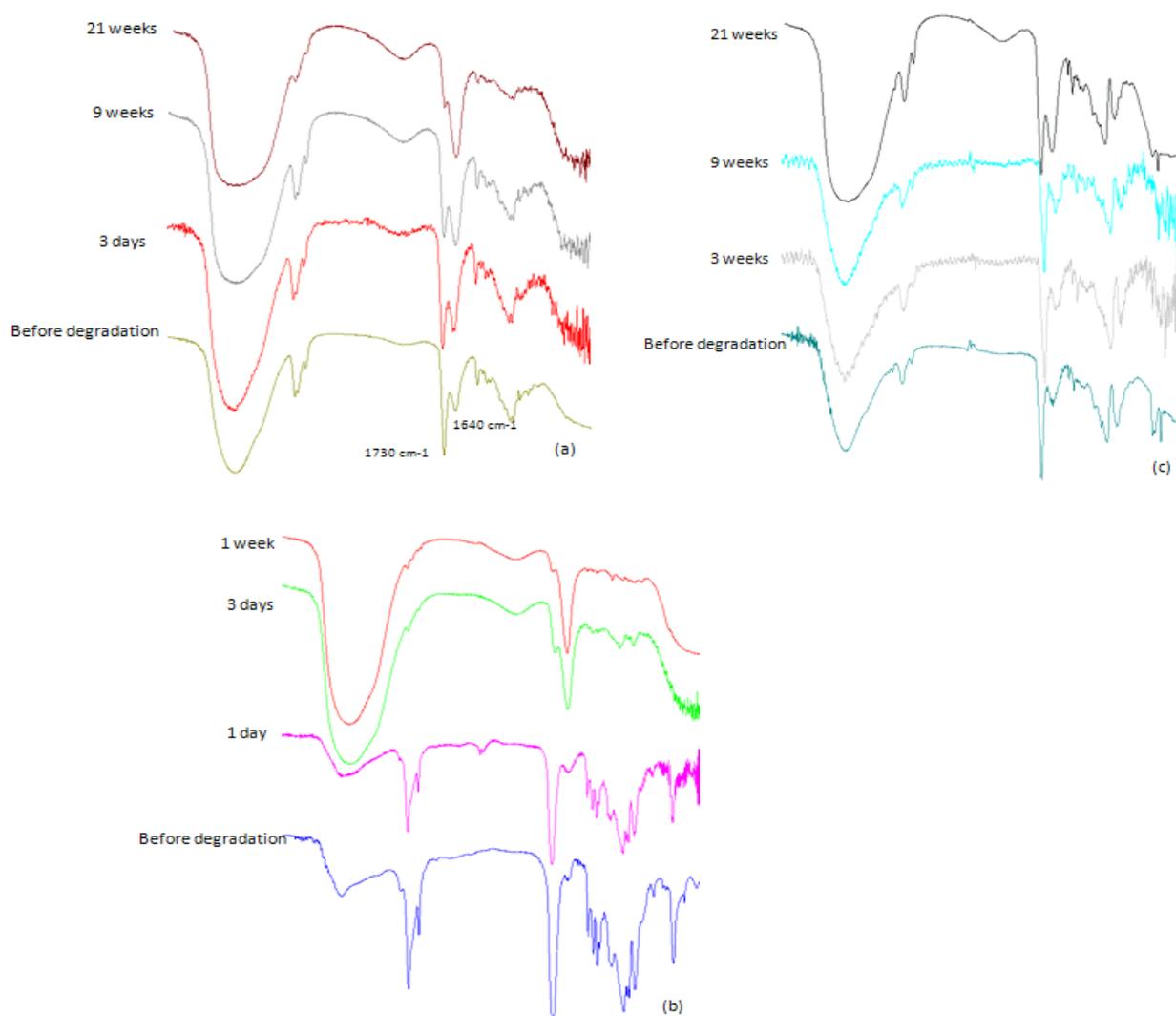
Inspection of the second stage of the degradation revealed that copolymers of HOPhe and HOLeu are quite resistant to buffer medium and are slowly hydrolyzed during a period of several months (k_2 from 0.02 to 0.3 week⁻¹, $t_{1/2}$ = 3 to 25 weeks), whereas copolymers of HOIle and HOVal have a much shorter life time of several days to weeks (k_1 from 1 to 5 week⁻¹, $t_{1/2}$ = 22 to 60 hours). For copolymers of CA (Table 1, compounds 20 and 23) their degradation time is extended (k_2 from 0.02 to 0.04 week⁻¹, $t_{1/2}$ = 16 to 21 weeks). However the degradation duration of copolymers of LA (Table 1, compounds 12 to 15) is similar to the homopolymers.

From Table 3, it is apparent that copolymers of CA are quite resistant to hydrolytic degradation and that in general these polymers discard only 70% (average) of their weight during five months. The inductive effect of the α -oxygen atom of the repeat unit in α -hydroxy polyesters enhances the reactivity of the ester carbonyl group and the acidity of the carboxylic end group, relative to the caproate ester structure [27]. Subsequently, the polyesters of α -hydroxy acid appeared to be much more sensitive to hydrolytic reaction than their corresponding caproate copolymers.

2.3.2. IR Analysis

During the degradation, the polymers were analyzed by IR spectroscopy, following the decrease of the ester band at $1730\text{--}1760\text{ cm}^{-1}$ and the increase of the carboxylic band at 1640 cm^{-1} . The ester signal decreases slowly while a carboxylic band develops simultaneously. It is worthy to note that the ester band does not vanish completely even at about 90% of weight loss. The IR spectra for several polymers along the degradation process are presented in Figure 5. PolyHOIle-CA (a) and PolyHOPhe-CA (b), like other copolymers of CA, exhibit a slow degradation pattern, and after 21 weeks the ester band is still present. PolyHOPhe-CA degrades much slower than PolyHOIle-CA, as is observed after nine weeks; the IR spectrum of PolyHOPhe-CA is almost identical to its initial spectrum (before degradation) whereas in the PolyHOIle-CA spectrum, the areas under the curves of the ester band and the carboxylic band are equal (1:1). PolyHOLEu-GA shows a rapid degradation rate and after a week the ester band nearly disappears.

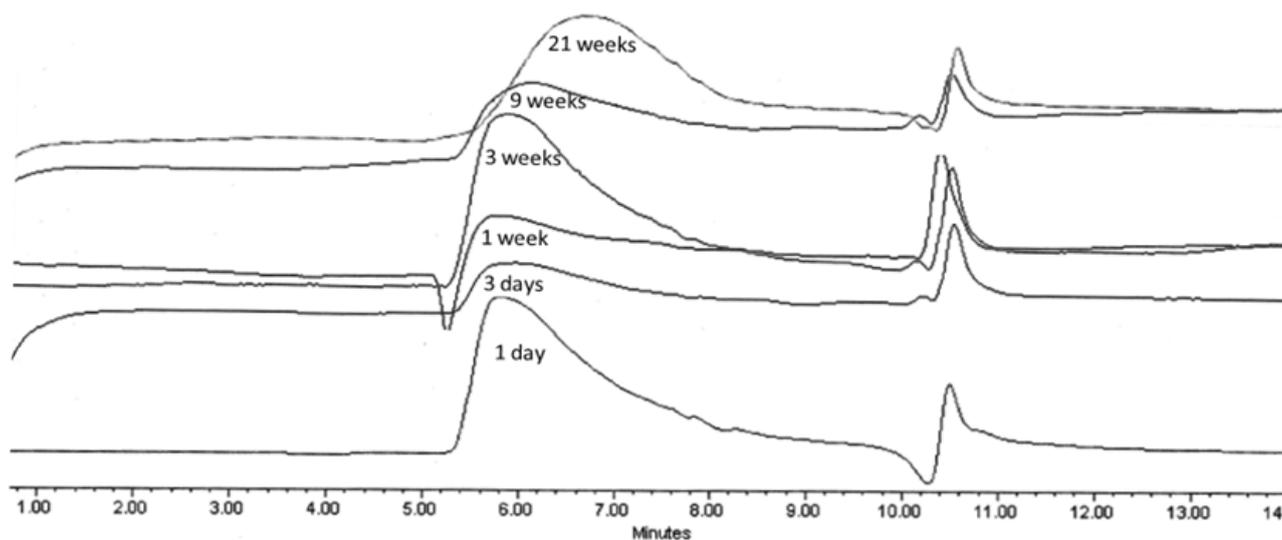
Figure 5. IR spectra of some polymers at several time points during degradation. (a) PolyHOIle-CA, (b) PolyHOLEu-GA. (c) PolyHOPhe-CA.



2.3.3. GPC Analysis

Molecular weight decrease was monitored by GPC analysis during the hydrolysis. Typical changes in the chromatograms during the course of the degradation process for PolyHOPhe-CA are illustrated in Figure 6. Through the first week the chromatograms look alike and there is no significant change in the elution time of the sample. A slight increase in the molecular weight could be noticed due to the dissolution of small oligomers into the buffer solution [28]. After three weeks of exposure to buffer, the chromatogram peak slowly moves to longer elution times. The molecular weight of the polymer starts to decrease.

Figure 6. GPC chromatograms of PolyHOPhe-CA during the degradation.



The molecular weight decrease data was fitted (by a non linear regression) to a first order reaction rate equation composed of either one or two exponential terms as is presented in Equation 2. The data for some polymers showed a biphasic behavior regarding the molecular weight change while other polymers exhibited a monophasic behavior.

$$MW_t = (A_T - A_1) \times e^{-k_1 t} + A_1 \times e^{-k_2 t} \quad (2)$$

MW_t is the molecular weight of the residual polymer at time t . A_T is the initial molecular weight of the polymer and A_1 is the molecular weight of the polymer at the end of the first step. k_1 and k_2 are the rate constants of the first and second steps. The parameters of all polymers are summarized in Table 4.

The Mw degradation curves are illustrated (in part) in Figure 7.

Inspection of Table 4 reveals that a significant number of polymers exhibited a biphasic behavior along the molecular weight decrease. The kinetic behavior of the molecular weight change was quite similar to that of the outlined weight loss, except for five polymers (PolyHOPhe-HOVal, PolyHOPhe-HOIlle, PolyHOLeu-HOIlle, PolyHOIlle-CA, and PolyHOVal-CA). These polymers demonstrated a biphasic behavior in the weight loss experiment and monophasic behavior in their molecular weight decrease. Their k_1 values were low (from 0.01 to 0.07 week⁻¹, $t_{1/2} = 9$ to 53 weeks), which is rather in the magnitude of k_2 . Their molecular weight decrease was a slow, monophasic process. It is likely that their molecular weight remains constant during the first phase of weight loss

(surface oligomers are hydrolyzed only), and the molecular weight decrease starts only towards the second phase of weight loss. For all other polymers, the magnitude of the rate constants for both processes was similar (weight loss and Mw decline), but the molecular weight decrease was slightly slower than the weight loss. The rate constants of the two processes are compared in Table 5.

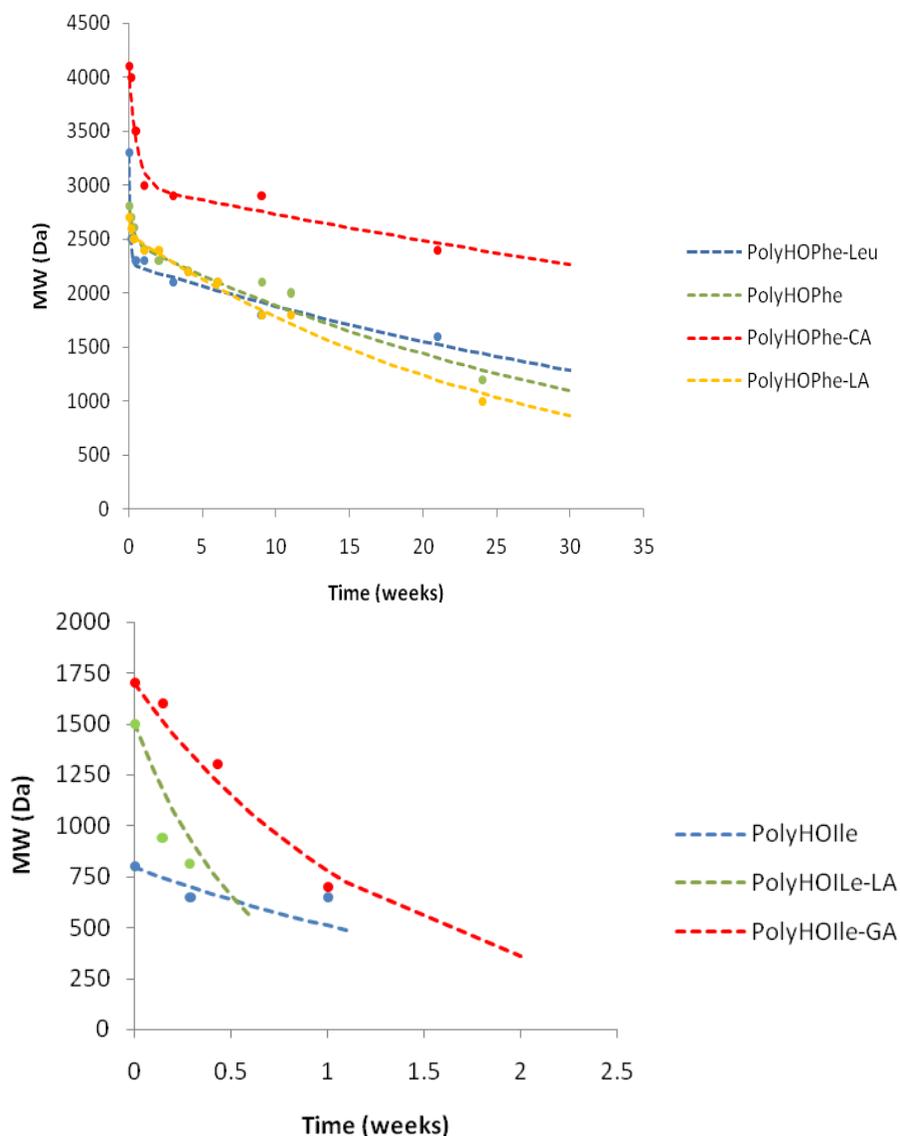
Table 4. Calculated rate constants (k_1 , k_2 , A01 and $t_{(1/2)}$) of molecular weight decrease emerging from fitting the experimental data to Equation 2. A01 is the percentage of the molecular weight decrease at the end of the first step of degradation ($A01 = (A_T - A_1) \div A_T \times 100$).

Polymer	$k_1(\text{week}^{-1})$ $t_{(0.5)\text{h}}$	A01	$k_2(\text{week}^{-1})$ $t_{(0.5)\text{weeks}}$	k_1/k_2
PolyHOPhe	3.03 38.04	11.2	0.027 25.6	105
PolyHOPhe-CA	1.87 62.2	27	0.0094 73.2	198
PolyHOPhe-GA	1.44 80.85	28.4	0.018 38.5	80
PolyHOPhe-LA	8.54 13.6	5.6	0.036 19.25	237
PolyHOPhe-Val	0.0738 9.4 weeks	–	–	–
PolyHOPhe-Ile	0.0176 39.4 weeks	–	–	–
PolyHOPhe-Leu	10.39 11.2	31	0.019 36.5	546
PolyHOLEu	8.157 14.2	31	0.059 11.7	138
PolyHOLEu-CA	1.26 92.4	39	0.0074 93.6	170.2
PolyHOLEu-GA	0.786 148	32.8	0.0113 61.3	69
PolyHOLEu-LA	8.085 14.4	9.4	0.0462 15	89.8
PolyHOLEu-Val	4.72 24.6	23	0.020 34.6	236
PolyHOLEu-Ile	0.0738 9.4 weeks	–	–	–
PolyHOIle	0.448 260	–	–	–
PolyHOIle-CA	0.045 15.4 weeks	–	–	–
PolyHOIle-GA	0.781 149	–	–	–

Table 4. Cont.

Polymer	$k_1(\text{week}^{-1})$ $t_{(0.5)\text{h}}$	A01	$k_2(\text{week}^{-1})$ $t_{(0.5)\text{weeks}}$	k_1/k_2
PolyHOIle-LA	1.656 70.3	–	–	–
PolyHOVal	0.395 294	–	–	–
PolyHOVal-CA	0.0131 53.0 weeks	–	–	–
PolyHOVal-GA	2.40 48.5	–	–	–
PolyHOVal-LA	1.145 119	–	–	–
PolyHOVal-Ile	0.327 356	–	–	–

Figure 7. Kinetic pattern of molecular weight decrease during hydrolysis.



During the burst stage in the weight loss course the main process seems to be the dissolution of small oligomers present in the polymer mass, which does not result in molecular weight decrease. This process can even potentially yield an increase in the molecular weight. During the second stage of weight loss, the polymer is cleaved but the weight loss remains faster than the change in molecular weight as shown in Table 5.

Table 5. Comparison between rate constants of weight loss and molecular weight change experiments.

	Weight Loss	MW Decrease
k_1	1.9 to 17.5 week ⁻¹	0.3 to 10 week ⁻¹
k_2	0.02 to 0.3 week ⁻¹	0.007 to 0.06 week ⁻¹
$t_{1/2}(1)$	6.7 to 60 hours	11 to 356 hours
$t_{1/2}(2)$	2.6 to 26 weeks	11 to 93 weeks

Generally, it was established that polyesters containing either HOVal or HOIle degraded to oligomers of less than 1000 Da within a few days ($k_1 = 0.4$ to 2.4 week⁻¹), independently of the initial molecular weight. This fast decrease is parallel to their rapid weight loss, which is monophasic. On the other hand, polyesters containing HOLEu and HOPhe exhibited significantly slower decreases in molecular weight in accordance with their weight loss behavior. The difference between these two groups might be due to the spatial orientation of the aliphatic chains/ring along the polymer. Copolymers of CA show a decrease in Mw during the first two or three weeks to attain a molecular weight of 2000–3000 Da, remaining at a constant value for the leftover period of the degradation experiment. The insertion of GA in the polymers accelerates the hydrolytic rate whereas LA does not significantly affect molecular weight decrease (or similarly, the weight loss).

2.3.4. NMR Analysis

Copolymers of HOPhe were monitored by NMR in order to determine the ratio between the two units in the polymer during degradation. For each copolymer, two peaks were selected in the NMR spectrum. One was assigned to unit A (-OPhe-) and the other was assigned to B (Table 6). At each time point, the ratio between A (-OPhe-) and B was calculated and the results are plotted in Figure 8.

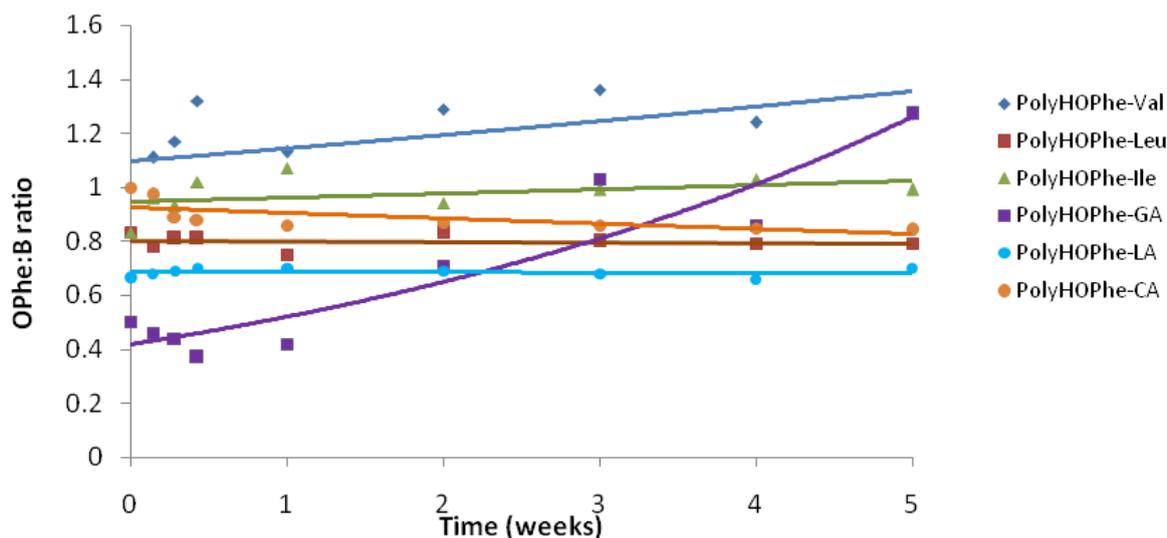
It is apparent that for PolyHOPhe-Leu and PolyHOPhe-LA, the two components (-OPhe-) and B(-OLEu and -OLA) were released at the same rate (horizontal lines) and there is no change in the polymer composition during the degradation. On the other hand, the ratio between HOPhe and GA grew during the degradation process, meaning that GA was evacuated faster from the polymer than HOPhe. This is also the case with PolyHOPhe-Val and PolyHOPhe-Ile, although the change was less pronounced. An inverse trend can be observed for PolyHOPhe-CA, in which the HOPhe unit was released faster to the buffer medium than the CA one.

It is conceivable to assume that a unit that is cleared faster from the polyester is more sensitive to hydrolytic degradation due to its hydrophilicity (GA) or side chain orientation (HOVal and HOIle). CA is less susceptible to hydrolysis than HOPhe as a result of the α -hydroxy acid effect explained above. The change in polymer composition affects the profile of the hydrolysis.

Table 6. NMR peaks corresponding to unit A (-OPhe-) and B in the copolymers PolyHOPhe-B and their initial ratios.

Copolymer	Chemical shift of Peak A (HOPhe, ppm)	Chemical shift of Peak B (ppm)	A:B at t = 0
PolyHOPhe-Ile	7.26 (5 aromatic protons)	0.92 (6 methyl protons)	5:6
PolyHOPhe-Leu	7.26 (5 aromatic protons)	0.93 (6 methyl protons)	5:6
PolyHOPhe-Val	7.26 (5 aromatic protons)	0.85 (6 methyl protons)	5:6
PolyHOPhe-LA	3.18 (2 CH ₂ protons)	1.52 (3 methyl protons)	2:3
PolyHOPhe-GA	5.32 (1 CH proton)	4.72 (2 CH ₂ protons)	1:2
PolyHOPhe-CA	3.18 (2 CH ₂ protons)	2.29 (2 CH ₂ protons)	1:1

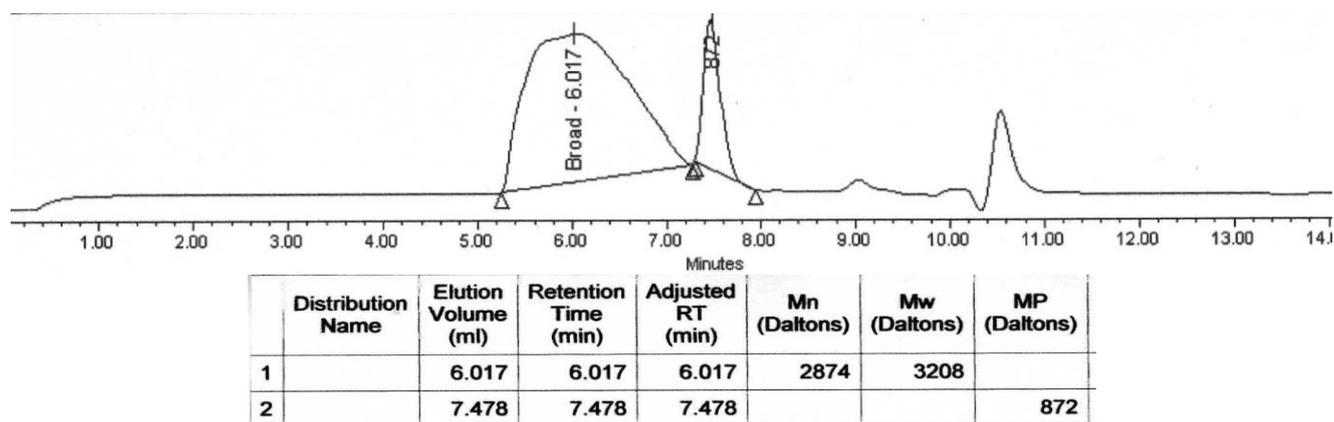
Figure 8. Composition of the HOPhe-B copolymers during degradation as been derived from NMR data.



2.3.5. Buffer Solutions Analysis

The buffer solutions were found to contain, on the one hand, part of the polymers that were likely torn off from the surface to the buffer, and on the other hand, oligomers that were cleaved from polymer chain and dissolved in the buffer (Figure 9). The oligomers that were found in GPC chromatograms have a molecular weight between 600 and 900 Da. No smaller fractions were detected. This indicates that the smallest oligomers cleaved during the hydrolysis are tetramers since no monomers or dimers could be observed.

Figure 9. GPC chromatogram of buffer extracted oligomers of PolyHOPhe-CA after a week of degradation.



3. Experimental

3.1. Materials

Lactic acid (LA) was purchased from J. T. Baker, Deventer, Holland, and glycolic acid (GA) from Sigma-Aldrich, Milwaukee, WI. 6-hydroxyhexanoic (CA) was obtained from hydrolysis of ϵ -caprolactone, Acros Organics, Geel, Belgium. The amino acids were purchased from Fluka, Buchs, Switzerland and Sigma-Aldrich, Milwaukee, WI (98–99% pure). Sodium nitrite (99.5%) and p-Toluenesulfonic acid-monohydrate (98.5%) were also purchased from Sigma-Aldrich, Milwaukee, WI. All solvents were analytical-grade from either BioLab, Jerusalem, Israel; Frutarom, Haifa, Israel; and Gadot, Or Akiva, Israel, and were used without further purification.

3.2. Techniques

IR (2000 FTIR, PerkinElmer) measurements of thin polymers film was performed on NaCl plates employing a chloroform solution of the polymers. The molecular weights of the polyesters were estimated on a gel permeation chromatography (GPC) system consisting of a Waters 1515 isocratic HPLC pump with a Waters 2410 refractive index detector and a Rheodyne (Coatati, CA) injection valve with a 20 μ L-loop (Waters, Ma). The samples were eluted with CHCl_3 through a linear Styragel HR1 column (Waters) at a flow rate of 1 mL/min. The molecular weights were determined relative to polystyrene standards (Polyscience, Warrington, PA) with a molecular weight range of 100–5000 using the Breeze computer program. $^1\text{H-NMR}$ spectra (CDCl_3 for the polymers and DMSO-d_6 for the hydroxy acids) were obtained on a Varian 300MHz spectrometer in 5 mm o.d. tubes. The optical activity of the monomers and polymers was determined on a PE 343 polarimeter (PerkinElmer). Thermal behavior of the polymers was determined on a Mettler TA 4000-DSC differential scanning calorimeter (Mettler-Toledo, Schwerzenbach, Switzerland), calibrated with Indium standard heated at a rate of 10 $^\circ\text{C}/\text{min}$ under nitrogen atmosphere. Contact angles were measured with a Rame ́Hart model 100 contact angle goniometer on polymer covered microscope slides.

3.3. Synthesis of α -Hydroxy Acids

Sodium nitrite solution in water (NaNO_2 , 4.2 g in 15 mL, 60 mmol) was slowly added to the chilled amino acid (10 mmol) solution in 0.5 M sulfuric acid (40 mL, 20 mmol). Subsequently, the solution was slowly brought to room temperature and stirred overnight. Then, the aqueous solution was saturated with sodium chloride and extracted with ether. Extracts were combined, dried over Na_2SO_4 and the solvent removed. After lyophilization, the solid residue was recrystallized from ether-hexane mixture [12,13,29]. The hydroxy acids derived from L-Isoleucine, L-Leucine, L-Phenylalanine and L-Valine were synthesized.

(S)-2-hydroxy-3-methylpentanoic acid ((L)HO-Ile): $^1\text{H-NMR}$: 3.75 (1H, d), 1.65 (1H, m), 1.38 (1H, m), 1.12 (1H, m), 0.84 (3H, d), 0.81 (3H, t); Mp: 51 °C, $[\alpha]_D$ (c = 1.31, MeOH): +8.2, Yield: 65%

(S)-2-hydroxy-4-methylpentanoic acid ((L)HO-Leu): $^1\text{H-NMR}$: 3.91 (1H, dt), 1.73 (1H, m), 1.40 (2H, m), 0.86 (6H, dd); Mp: 78 °C lit. 78–80 °C [30], $[\alpha]_D$ (c = 1.67, MeOH): −9.6, Yield: 65%

(S)-2-hydroxy-3-phenylpropionic acid ((L)HO-Phe): $^1\text{H-NMR}$: 7.23 (5H, m), 4.14 (1H, dt), 2.87 (2H, dq); Mp: 123 °C lit. 123–124 °C [30], $[\alpha]_D$ (c = 1.50, MeOH): −16.1 lit. $[\alpha]_D$ = −20.0 (c = 2, H_2O) [29], Yield: 65%

(S)-2-hydroxy-3-methylbutanoic acid ((L)HO-Val): $^1\text{H-NMR}$: 3.71 (1H, d), 1.88 (1H, m), 0.86 (3H, d), 0.78 (3H, d); Mp: 60 °C, $[\alpha]_D$ (c = 1.58, MeOH): +4.4, Yield: 65%

3.4. Polymerization of the α -Hydroxy Acids

The hydroxy acids were polymerized by direct condensation [31,32]. In a typical experiment, 6.4–11.1 mmol (~1 g) of the hydroxy acid was suspended in toluene (3 mL) and 1% w/w (1 mg) of p-toluene sulfonic acid as catalyst was added. The solution was refluxed overnight in a Dean Stark apparatus. The toluene was then removed and the oligomers were treated in bulk at 150 °C, under nitrogen for 2 hours and then under a vacuum of 15 mm Hg for another 14–20 hours [16,31]. The co-polymers were prepared under the same experimental conditions where a molar ratio of 1:1 was applied to the various hydroxy acid monomers, leading to random copolymers. Homopolymers from (L)lactic acid, (L)HOIle, (L)HOLeu, (L)HOPhe and (L)HOVal and copolymers of these hydroxy acids with lactic acid, glycolic acid and 6-hydroxyhexanoic acid were synthesized. The polymers were used as they are. The polymers were characterized by $^1\text{H-NMR}$, IR, GPC, Optical activity, and solubility in several solvents. The characterization data of these polymers is summarized in Table 1.

3.5. Toxicity

The synthesized polymers were evaluated for biocompatibility in regular culture conditions upon coating cover glass with the polymers at concentrations of 1–100 $\mu\text{g/mL}$ (dissolved in chloroform). The cover glasses were then dried under vacuum and washed, and inserted into conventional 24-well tissue culture plates. Thereafter the plates were exposed to UV light for 15 min and were further used

for cell culturing. PC12 pheochromocytoma neuronal cell line and bEnd.3 brain endothelial cell (EC) line were plated and maintained in culture conditions as previously described [33], and followed-up for adherence, proliferation and differentiation for seven days using Alamar blue and lactate dehydrogenase assays.

3.6. In vitro Hydrolytic Degradation of the Polymers

Samples (~200 mg) of the dry polymers were injected into 50 mL phosphate buffer solution pH 7.4, 0.1 M. The degradation was conducted at physiological conditions (37 °C, 100 rpm). The buffer solution was replaced every 24 h during the first week, and every week afterwards to avoid saturation of the buffer solution. At each time point, the polymers were washed with water, lyophilized and weighed. Samples were taken from the polymers and analyzed. All experiments were done in duplicate. The degradation of the polymers was followed by weight loss, GPC, IR and NMR for three months. The weight loss was calculated as follows:

$$\%WL = \frac{A_0 - A_t}{A_0} \times 100 \quad (3)$$

A_0 is the polymer sample weight at the beginning of the experiment, and A_t is the sample weight at each time point of the experiment.

At each time point, buffer solution samples were acidified with HCl, extracted with chloroform, concentrated and checked by GPC.

4. Conclusions

New polyesters derived from α -amino acids were synthesized and investigated for their toxicity and degradation, followed by the composition of the degraded polymers and the oligomers released into the buffer medium. The polymers were found to be non toxic for two cell lines. Most polymers exhibit a biphasic degradation behavior, where the initial phase is fast while the second phase is much slower. The first phase of degradation is related to surface interactions with the buffer medium whereas the second phase is related to bulk interactions. In most cases, a linear relationship was found between the weight loss constant and the hydrophobicity of the polymers.

The polyesters exhibit quite different characteristic patterns during hydrolysis. Some are degraded quickly within a few days to weeks whereas others are resistant for several months. Thus, these hydroxy acids and their derived polyesters offer a wide range of biocompatible materials and have good potential for medical applications such as drug delivery and drug carriers. These hydroxy acids are now being polymerized to high molecular weight polyesters by other methods and are tested for degradation (to be published elsewhere).

Acknowledgments

This study was supported by The Israel Science Foundation (grant No. 591/08) and the Alex Grass Center for Drug Design and synthesis of Novel Therapeutics.

References

1. Albertsson, A.C. Degradable Aliphatic Polyesters. In *Advances in Polymer Science*; Springer-Verlag: Berlin/Heidelberg, Germany, 2002; Volume 157.
2. Li, S.M.; McCarthy, S. Further investigations on the hydrolytic degradation of poly(DL-lactide). *Biomaterials* **1999**, *20*, 35–44.
3. Vert, M. Aliphatic polyesters: Great degradable polymers that cannot do everything. *Biomacromolecules* **2005**, *6*, 538–546.
4. Huang, M.H.; Li, S.M.; Hutmacher, D.W.; Coudane, J.; Vert, M. Degradation characteristics of poly(epsilon-caprolactone)-based copolymers and blends. *J. Appl. Polym. Sci.* **2006**, *102*, 1681–1687.
5. Hutmacher, D.W. Scaffold design and fabrication technologies for engineering tissues—State of the art and future perspectives. *J. Biomater. Sci. Polym. Ed.* **2001**, *12*, 107–124.
6. Hutmacher, D.W.; Schantz, T.; Zein, I.; Ng, K.W.; Teoh, S.H.; Tan, K.C. Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. *J. Biomed. Mater. Res.* **2001**, *55*, 203–216.
7. Li, S.V.M. Biodegradation of Aliphatic Polyesters. In *Degradable Polymers: Principles and Applications*, 2nd ed.; Scott, G., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2002.
8. Pitt, C.G. Biodegradable Polymers as Drug Delivery Systems. In Chasin, M., Langer, R., Ed. Marcel Dekker: New York, 1990.
9. Vert, M.C.P.; Chabot, F.; Leray, J. *Macromolecular Biomaterials*; Hastings, G.W., Ducheyne, P, Eds.; CRC Press: Boca Raton, FL, USA, 1984.
10. Benabdillah, K.M.; Coudane, J.; Boustta, M.; Engel, R.; Vert, M. Synthesis and characterization of novel degradable polyesters derived from D-gluconic and glycolic acids. *Macromolecules* **1999**, *32*, 8774–8780.
11. Slager, J.; Domb, A.J. Biopolymer stereocomplexes. *Adv. Drug Deliv. Rev.* **2003**, *55*, 549–583.
12. Deechongkit, S.; You, S.L.; Kelly, J.W. Synthesis of all nineteen appropriately protected chiral alpha-hydroxy acid equivalents of the alpha-amino acids for Boc solid-phase depsi-peptide synthesis. *Org. Lett.* **2004**, *6*, 497–500.
13. Bauer, T.; Gajewiak, J. alpha-Hydroxy carboxylic acids as ligands for enantioselective diethylzinc additions to aromatic and aliphatic aldehydes. *Tetrahedron* **2004**, *60*, 9163–9170.
14. Antheunis, H.; van der Meer, J.C.; de Geus, M.; Kingma, W.; Koning, C.E. Improved Mathematical Model for the Hydrolytic Degradation of Aliphatic Polyesters. *Macromolecules* **2009**, *42*, 2462–2471.
15. Antheunis, H.; van der Meer, J.C.; de Geus, M.; Heise, A.; Koning, C.E. Autocatalytic Equation Describing the Change in Molecular Weight during Hydrolytic Degradation of Aliphatic Polyesters. *Biomacromolecules* **2010**, *11*, 1118–1124.
16. Albertsson, A.C.; Varma, I.K. Aliphatic polyesters: Synthesis, properties and applications. In *Degradable Aliphatic Polyesters*; Springer: Berlin, Germany, 2002; Volume 157, pp. 1–40.
17. Mark, J.E. *Polymer Data Handbook*; Oxford University Press: New York, NY, USA, 1999; Volume 1.

18. Fukuzaki, H.; Yoshida, M.; Asano, M.; Kumakura, M.; Imasaka, K.; Nagai, T.; Mashimo, T.; Yuasa, H.; Imai, K.; Yamanaka, H. Synthesis of Biodegradable Copoly(L-Lactic Acid/Aromatic Hydroxyacids) with Relatively Low-Molecular-Weight. *Eur. Polym. J.* **1990**, *26*, 1273–1277.
19. Cohen-Arazi, N.; Katzhendler, J.; Domb Abraham, J.; Hagag, I.; Kolitz, M.; Arien-Zakay, H.; Lazarovici, P.; Lecht, S. Biodegradable Polyester Matrixes Derived from α -Hydroxy Acids as Cell Biocompatible Substratum. **2010**, in preparation.
20. Simitzis, J.; Triantou, D.; Soulis, S.; Triantou, K.; Simitzis, C.; Zoumpoulakis, L. Correlation of hydrolytic degradation with structure for copolyesters produced from glycolic and adipic acids. *J. Mater. Sci. Mater. Med.* **2010**, *21*, 1069–1079.
21. Lee, J.W.; Gardella, J.A.; Hicks, W.; Hard, R.; Brigh, F.V. Analysis of the Initial Burst of Drug Release Coupled with Polymer Surface Degradation. *Pharm. Res.* **2003**, *20*, 149–152.
22. Zaikov, L.V.S. The Mechanism of Chemical Degradation of Polymers: Part I: Quantitative Prediction of Hydrolytic Stability of Polyesters. *Polym. Degrad. Stabil.* **1984**, *9*, 41–50.
23. Gopferich, A. Polymer bulk erosion. *Macromolecules* **1997**, *30*, 2598–2604.
24. Fukuzaki, H.; Yoshida, M.; Asano, M.; Kumakura, M. Synthesis of Copoly(D,L-Lactic Acid) with Relatively Low Molecular Weight and *in vitro* Degradation. *Eur. Polym. J.* **1989**, *25*, 1019–1026.
25. Panyam, J.; Dali, M.M.; S.K., S.; Ma, W.; Chakravarthi, S.S.; Amidon, G.L.; Levy, R.J.; Labhasetwar, V. Polymer degradation and *in vitro* release of a model protein from poly(D,L-lactide-co-glycolide) nano- and microparticles. *J. Control. Release* **2003**, *92*, 173–187.
26. Tang, Z.G.; Black, R.A.; Curran, J.M.; Hunt, J.A.; Rhodes, N.P.; Williams, D.F. Surface properties and biocompatibility of solvent-cast poly[epsilon-caprolactone] films. *Biomaterials* **2004**, *25*, 4741–4748.
27. Pitt, C.G.; Gratzl, M.M.; Kimmel, G.L.; Surles, J.; Schindler, A. Aliphatic polyestersII. The degradation of poly (DL-lactide), poly (E-caprolactone), and their copolymers *in vivo*. *Biomaterials* **1981**, *2*, 215–220.
28. Schliecker, G.; Schmidt, C.; Fuchs, S.; Wombacher, R.; Kissel, T. Hydrolytic degradation of poly(lactide-co-glycolide) films: effect of oligomers on degradation rate and crystallinity. *Int. J. Pharm.* **2003**, *266*, 39–49.
29. Shin, I.; Lee, M.R.; Lee, J.; Jung, M.; Lee, W.; Yoon, J. Synthesis of optically active phthaloyl D-aminoxy acids from L-amino acids or L-hydroxy acids as building blocks for the preparation of aminoxy peptides. *J. Org. Chem.* **2000**, *65*, 7667–7675.
30. Degerbeck, F.; Fransson, B.; Grehn, L.; Ragnarsson, U. Synthesis of N-15-Labeled Chiral Boc-Amino Acids from Triflates—Enantiomers of Leucine and Phenylalanine. *J. Chem. Soc. Perk. Trans. 1* **1993**, *1*, 11–14.
31. Kajiyama, T.; Kobayashi, H.; Taguchi, T.; Kataoka, K.; Tanaka, J. Improved synthesis with high yield and increased molecular weight of poly(alpha,beta-malic acid) by direct polycondensation. *Biomacromolecules* **2004**, *5*, 169–174.
32. Simmons, T.L.; Baker, G.L. Poly(phenyllactide): Synthesis, characterization, and hydrolytic degradation. *Biomacromolecules* **2001**, *2*, 658–663.

33. Lecht, S.; Arien-Zakay, H.; Wagenstein, Y.; Inoue, S.; Marcinkiewicz, C.; Lelkes, P.I.; Lazarovici, P. Transient signaling of Erk1/2, Akt and PLCgamma induced by nerve growth factor in brain capillary endothelial cells. *Vas. Pharmacol.* **2010**, *53*, 107–114.

© 2010 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).