Degradation and analysis of synthetic polymeric materials for biomedical applications
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Chapter 3

3. Monitoring the *in vitro* enzyme-mediated degradation of degradable poly(ester amide) for controlled drug delivery by LC-ToF-MS

To scrutinize materials for specific biomedical applications, we need sensitive and selective analytical methods that can give more insight into the process of their biodegradation. In the present study, the enzymatic degradation of multi-block poly(ester amide) based on natural amino acids, such as lysine and leucine, was performed with serine proteases (α-chymotrypsin (α-CT) and proteinase K (PK)) in phosphate-buffered saline solution at 37°C for 4 weeks. Fully and partially degraded water-soluble products were analyzed by liquid chromatography hyphenated with time-of-flight mass spectrometry using an electrospray interface (LC-ESI-ToF-MS). Tracking the release of monomeric and oligomeric products into the enzyme media during the course of enzymatic degradation revealed the preferences of R-CT and PK toward ester and amide bonds: both α-CT and PK showed esterase and amidase activity. Although within the experimental time frame up to 30 and 15% weight loss was observed in case of α-CT and PK, respectively, analysis by size-exclusion chromatography showed no change in the characteristic molecular-weight averages of the remaining polymer. This suggests that the enzymatic degradation occurs at the surface of this biomaterial. A sustained and linear degradation over a period of 4 weeks supports the potential of this class of poly(ester amide)s for drug delivery applications.

1 Introduction

The demand for design and synthesis of biodegradable polymeric materials for application in drug-delivery devices, scaffolds for tissue engineering, and surgical implants, such as sutures, pins, rods, and screws for fixation devices, is increasing [1,2]. From this perspective, degradable poly(ester amide)s (PEAs), preferably those containing natural amino acids, have gained much attention. These materials have been studied extensively in terms of their biocompatibility and degradation and have been tested in vivo as drug-eluting stent coatings in clinical trials such as the NOBLESSE trial [3,4]. PEAs contain ester bonds that are susceptible to hydrolytic degradation. The inclusion of α-amino acids results in improved mechanical and thermal properties through hydrogen bond interactions [5]. In general, degradation of biomaterials may take place either through bulk degradation or via surface erosion [6]. It is important to differentiate between these two mechanisms for a given biomaterial if one is to control the drug release rate by changing the ratios of ester, amide, and methylene groups. This requires knowledge of the relative rates of hydrolysis at different sites within the polymeric chains and determination of the structure of degradation products, which may critically affect the biocompatibility and rate of clearance from the body [7].

The sequence of the monomers in a multi-block polymer affects its crystalline or amorphous nature, which in turn influences the degradation rate [8,9]. Therefore, it is important to separate and identify the low-molecular-weight water-soluble products during biodegradation in order to correlate the structural parameters with rates of degradation [10]. The collected information will be helpful (i) to estimate overall structure and optimize the synthetic approach towards a functional material, (ii) to evaluate the toxicity of the degradation products at an early stage, (iii) to determine the rate of hydrolysis at different sites (e.g., specificity of enzyme for ester or amide bonds) and (iv) for the rational design of new materials.

Several studies report the use of liquid chromatography for the separation of water-soluble degradation products of polyesters and their identification with UV-Vis using standards [11], FAB-MS [12], ESI-MS [13], and so on or their offline analysis with NMR [14]. Rizzarelli et al. utilized the combination of high-performance liquid chromatography with electrospray-ionization mass spectrometry (LC-ESI-MS) or tandem mass spectrometry (LC-ESI-MS/MS). They noticed selective ester hydrolysis in aliphatic copolyesters catalyzed by lipases [15]. Mass spectrometry has emerged as a powerful analytical tool for the characterization of natural and synthetic macromolecules [7,16], but the technique has limitations for high-
molecular-weight synthetic polymers. This is true for both ionization techniques that are commonly applied for high-molecular-weight analytes, that is, ESI and matrix-assisted laser desorption/ionization (MALDI). When using ESI, multiply charged ions swamp the spectrum, amplifying the number of ions arising from the molecular-weight distribution (MWD). In MALDI, both statistics and charge affinity may cause low-molecular-weight oligomers to dominate the spectrum.

In the current chapter, stainless steel disks coated with a class of PEAs (Scheme 1) composed of sebacic acid, 1,6-hexanediol, lysine, and leucine with two different sequences of repeating units were subjected to \textit{in vitro} enzymatic degradation with \(\alpha\)-chymotrypsin (\(\alpha\)-CT) and proteinase K (PK). The weight-average molecular weight (\(M_w\)) and dispersity of the remaining un-dissolved material were analyzed by SEC before and after degradation. The water-soluble degradation products (monomers and oligomers) were separated on an LC column and their structure was characterized by time-of-flight (ToF) MS.

The objectives of the current study were (i) to determine the effectiveness of \(\alpha\)-CT and PK as model enzymatic systems to degrade the present PEA by measuring weight losses, (ii) to differentiate between surface and bulk degradation by estimating the average molecular weights and dispersity of the polymer before and after degradation, and (iii) to assess the specific activity (qualitative assessment of preferences and reaction rates) of the enzymes with the present PEA by (semi-quantitatively) determining the monomeric and oligomeric products released during biodegradation.

The implication of this research is that a comparative degradation study of several PEAs by model enzymes may guide the development of PEA biomaterials towards specific properties, such as degradation rate, controlled drug-delivery rate, targeted release of active pharmaceutical molecules, and so on.

\textbf{Scheme 1} Structure of poly (ester amide)s subjected to enzymatic degradation. Where \(x = 3\) and \(y = 4\). The solid and dashed arrows represent the possible cleavage of ester and amide bonds, respectively.
Chapter 3

2 Materials and methods

2.1 Materials

The polymer was synthesized at DSM Ahead following a published procedure [17]. The chemical structure is shown in Scheme 1. The actual values are m/n = 3:1, x = 3, and y = 4. Figure 1 shows the $^1$H NMR spectrum of the PEA recorded in d$_6$-ethanol (Euriso-top, Gif sur Yvette, France) on a Varian Inova 500 MHz NMR (Varian, Walnut Creek, CA, USA) equipped with a 5 mm 13C/31P/1H GS Probe 500. A pulse-repetition time of 25 s, a pulse duration of 3.6 µs, 63 scans and a temperature of 25°C were used to record $^1$H spectra. The integration of characteristic protons signals for 1,6-hexanediol (15) and lysine (5) confirmed the molar ratio of 3:1 between $m$ and $n$ block of the polymer. There is 1:1 ratio between lysine (5) and benzyl group (18). Lysine (5) and sebacic acid (6) possess 1:1.13 ratio in the $n$ block of the polymer. The 1,6-hexanediol (15) and sebacic acid (6 + 9) signal ratio also supports the m/n = 3:1 molar ratio. Accurate integration was difficult in case of overlapping signals. The HSQC NMR experiments were done to confirm the characteristic signals (see Figure S1 in the supporting information). Glass transition temperature ($T_g$) of the analyzed polymer is 33°C, determined via DSC. The test sample, 5 mg, was vacuum-dried at 65 ± 5°C and placed in a crucible pan. Next the sample was analyzed on a Mettler Toledo DSC 822e instrument and $T_g$ was derived from the second heating curve.

2.2 Solubility

The solubility of the biomaterial in water and in a number of common organic solvents was assessed by combining 2 to 3 mg of the PEA with 1 mL of the respective solvents (Table 1) at room temperature (25°C). The PEA was considered to be soluble when the solutions became completely transparent.

2.3 Enzyme activity

$N$-Suc-Ala-Ala-Pro-Phe-pNA (Bachem, Bubenhof, Switzerland) was used as a chromogenic substrate to determine the activity of $\alpha$-CT from bovine pancreas (Fluka, Steinheim, Germany, pr.no. 27270, ≥ 68 units/mg protein). The amount of p-nitrophenyl anilide released was determined by recording the absorbance at 410 nm and 25°C using a
spectrophotometer. The amount of enzyme activity releasing 1 µmol of chromophore per min is defined as 1 unit [18].

The activity of the PK from Tritirachium album (Sigma Aldrich, Steinheim, Germany, pr. no. P2308, ≥30 units/mg protein) was measured by colorimetric analysis according to the quality-control procedure described by the supplier (Enzymatic Assay of Proteinase K, EC 3.4.21.64). One unit will hydrolyze urea-denatured hemoglobin to produce a colour equivalent to 1 µmol (181 µg) of tyrosine per min at pH 7.5 at 37 °C [19].

Figure 1 ¹H NMR spectrum of the PEA in d₆-ethanol.

2.4 In vitro enzyme-mediated degradation

Approximately 20 mg of PEA were drop-cast on one side of stainless-steel round disks (diameter 13 mm, thickness 80 µm, surface area 133 mm²). The coatings were applied in three layers by pipetting 70 µL of polymer solution prepared in ethanol (0.1 g/mL, filtered through a 0.45 µm filter). Each layer was allowed to air-dry for at least 2 h at ambient temperature before the next layer was applied. After the final layer was applied, the coated disks were air-dried overnight at room temperature, followed by drying at 40°C under reduced pressure to a constant weight.
Chapter 3

The coated dried disks (in triplicate) were immersed in 1.5 mL of phosphate-buffered saline (PBS) buffer (0.2 g KCl, 0.2 g KH$_2$PO$_4$, 1.15 g Na$_2$HPO$_4$, 8 g NaCl in 1 L, containing sodium azide (0.5 g/L, to inhibit bacterial growth) with α-CT (17 U/mL; pH ≈ 8) or with PK (5 U/mL; pH ≈ 7.4) in 15 mL polypropylene conical tubes (BD, Franklin Lakes, NJ, USA) and incubated at 37°C and 120 rpm (Innova 44 Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA). The enzyme solutions were refreshed every 48 h and stored at −20°C for LC-ToF-MS analysis after their pH was monitored. The remaining polymer samples (on disks, each in triplicate) were collected for gravimetric analysis randomly on day 7, 14, 21, and 28. The solutions were aspirated and the disks were rinsed three times for 5 min with distilled water. The samples of remaining polymer were then dried under vacuum at 65°C for 48 h. The samples were dried for additional 24 h at 65°C to ensure constant weight. The percent weight loss was calculated using the following formula:

\[
\text{Weight loss (\%)} = \left[1 - \frac{(\text{weight of the sample} + \text{disk}) - (\text{weight of the disk})}{(\text{initial weight of sample} + \text{disk}) - (\text{weight of the disk})}\right] \times 100
\]

Enzyme blank and polymer blank samples were also incubated and analyzed to correct the data. Under the current experimental setup, the enzyme media was replaced with fresh enzyme after every 48 h. Therefore, to estimate the decrease in the activity of enzymes, we collected samples from the enzyme media (both α-CT and PK) incubated with coated and non-coated disks after 8, 24, 32, and 48 h for LC-ToF-MS analysis.

2.5 Size-exclusion chromatography

The SEC experiments were performed on an LC system equipped with a LC-10AD solvent delivery module, a CTO-6A column oven, a SIL-9A auto injector, an SPD-10AV$_{vp}$ UV-vis detector, and an RID-10A refractive-index detector (all from Shimadzu, Kyoto, Japan). The SEC analyses were performed on three PLgel MIXED-B columns (10 µm, 300 × 7.6 mm i.d.; Polymer Laboratories, Church Stretton, U.K.) connected in series. THF stabilized with butylated hydroxytoluene (BHT) (BioSolve, Valkenswaard, The Netherlands) was pumped at a flow rate of 1 mL min$^{-1}$. The injection volume was 50 µL, and the column oven temperature was set at 50°C. Polystyrene standards (Polymer Laboratories, Shropshire, U.K.) were used to calibrate the SEC-UV-dRI system. Data were recorded and chromatographic
peaks were treated using Class-VP 7.4 software (Shimadzu). Molar-mass distributions (MMDs) were calculated from the chromatograms using software written in-house in Excel 2003 (Microsoft).

2.6 LC-ToF-MS study

We injected 5 µL of the enzyme solutions collected after every 48 h without dilution after filtration through a 0.2 µm pore size PTFE filter (Grace Davison). The stock solutions of 1,6-hexanediol (Aldrich), sebacic acid (Fluka) and benzyl alcohol (Fluka), were prepared in PBS, and quantification was based on a standard-addition method in positive ESI mode for 1,6-hexanediol and in negative ESI mode for sebacic acid. The concentration of benzyl alcohol was determined by LC-UV at 254 nm.

The chromatographic separations of soluble degraded products were performed on a Prevail C18 Column (250 × 4.6 mm i.d., 5 µm particle size) (Grace Davison, Deerfield, IL, USA) connected to an Agilent 1100-series LC system consisting of a degasser, a gradient pump, and an auto-sampler (all from Agilent Technologies, Waldbronn, Germany). The column oven (Temperature Control Module from Waters, Milford, MA, USA) was set at 40°C. Mobile phase A was 0.1% (v/v) aqueous formic acid (Fluka, Steinheim, Germany) and B was acetonitrile (Biosolve). The gradient was started at t = 0 min with 5% (v/v) B, reaching 60% (v/v) B in 25 min, held constant for 2 min, and then back to 5% (v/v) at 30 min (t_end = 35 min) at a flow rate of 1.5 mL min⁻¹ and was split between a waste reservoir and the ESI interface by means of a zero-dead-volume T-piece to ensure a flow of approximately ~0.2 mL min⁻¹ into the electrospray. Organic solvents used for the LC mobile phase were of LC grade. Highly pure water for mobile-phase preparation was obtained from an Arium 611 Ultrapure (18.2 MΩ*cm) Water System (Sartorius, Goettingen, Germany).

The LC system was hyphenated with a 6210 series time-of-flight mass spectrometer (Agilent) via an ESI interface. The conditions of the ESI-ToF-MS were as follows. Drying gas was nitrogen at 8 L min⁻¹, 300°C and 200 kPa. The capillary voltage was 3500 V; the fragmenter was set at 140 V and the skimmer at 60 V. Octopole dc1 was set at 33 V; octopole radio frequency at 250 V. The data were acquired in the scan mode from m/z 50 to 3000 with 0.88 scans/s. An Agilent MassHunter Workstation A.02.01 and Analyst QS 1.1 software (Applied Biosystems, Carlsbad, California, USA) were used for data acquisition and data analysis, respectively.
The LC-ToF-MS separation and identification of the water-soluble degradation products was optimized by monomers and oligomers, generated by means of chemical degradation. (see the supporting information).

3 Results and discussion

3.1 Solubility

The solubility of the PEA in a number of organic solvents at room temperature (25°C) is summarized in Table 1. The PEA is completely soluble in most of the polar (protic and aprotic) solvents, except in water, acetonitrile, and ethyl acetate.

<table>
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<th>Solubility</th>
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<tr>
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<td>+</td>
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<tr>
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<td>+</td>
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<td>Acetone</td>
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</tbody>
</table>

Soluble +, insoluble -

3.2 Overall effectiveness of in vitro enzyme-mediated degradation

The results in Figure 2 show a greater weight loss (30%) in case of α-CT than when using PK (15%) after 4 weeks under the current experimental conditions. When incubating with only PBS buffer, no noticeable weight loss was observed. The biomaterial showed a sustained and near-linear degradation over a period of 4 weeks (Figure 2). This weight loss the polymer indicates constant erosion of the surface by the enzymes. No solid particles were observed in the reaction media. No decrease in the pH of the degradation media was observed during the course of the enzyme-mediated degradation, implying that the erosion does not result in an accumulation of acidic degradation products.
Monitoring the in vitro enzyme-mediated degradation of degradable PEA by LC-ToF-MS

**Figure 2** Percentage of polymer weight loss as a function of the degradation time (days) in α-Chymotrypsin (α-CT, 17 U/mL) and proteinase K (PK, 5 U/mL) in PBS. The “blank” experiment in PBS is shown for comparison. Lines with triangles, squares and diamonds correspond to blank, PK and α-CT experimental data, respectively.

**Figure 3** The molar-mass distribution of the PEA before and after enzymatic degradation with α-CT. The side bar on the right side represents the number of days of treatment.
3.3 Molecular-weight of remaining material

The $M_n$ and dispersity of the starting material were 31 kDa and 2.1, respectively. The molecular weight and molecular-weight distribution (MWD) of the sample prior to degradation and of the remaining un-dissolved samples after incubation with α-CT were determined. The SEC-dRI system was calibrated with polystyrene standards in THF. No significant differences were observed between the molecular weights of the samples prior to and after enzymatic degradation (Figure 3). This maintenance of molecular weight is in line with the enzymatic degradation being a surface-erosion phenomenon. Similar results were reported by Fan et al. [20,21] and by Guo et al. [22].

3.4 LC-ToF-MS analysis following enzymatic degradation

The chromatographic separation and identification of the monomers and oligomers liberated as a result of enzymatic degradation was monitored using LC-ToF-MS with electro-spray ionization in the positive and negative modes. Formic acid was used to make proton adducts. Sodium adducts were also observed because of the presence of sodium ions in the incubating medium. The presence of multiple charge ions were identified by the differences of m/z values between the isotopic peaks of respective masses. The negative mode spectra showed a difference of 2 and 24 Da for proton and sodium adducts, respectively, as compared with positive mode spectra. Benzyl alcohol showed the [M-OH]$^+$ adduct only in positive mode. The m/z and the retention time of benzyl alcohol in the enzyme solutions were confirmed by the benzyl alcohol standard. The ESI-ToF-MS spectra of the identified peaks (Table 1) are presented in the supporting information. The copolymer consists of five components; leucine (L), lysine (K), 1,6-hexanediol (C6), sebacic acid (C10), and benzyl alcohol (BnOH). All the LC10 and KC10 combinations contained an amide bond. In case of LC6, the carboxylic group of L is connected to C6 via an ester bond. The carboxylic group of the lysine is end-capped with benzyl alcohol and forms an ester bond. Scheme 1 shows the possible fragmentation pattern in case of both ester- and amide-bond cleavage. If only the ester bonds are hydrolyzed, degradation products will be C6 (peak number 4), benzyl alcohol (6), LC10L (11), LC10LC6 (17), and the oligomers LC10(KC10)$_n$L originating from $n$ block of the polymer. If amide-bond cleavage is simultaneously taking place along with ester hydrolysis, then the additional products that may appear are K (peak number 1), L (3), LC6, C10 (8), LC10 (9), C10K (5), LC10K(7), and LC10KC10 (10).
Figure 4 displays the separation of the different degradation products at various intervals of time during enzymatic degradation of PEA with α-CT. Mass-spectrometric analysis shows the release of L (peak number 3), C6 (4), C10K (5), benzyl alcohol (6), LC10K (7), C10 (8) LC10 (9) LC10KC10 (10), LC10L (11). Peaks 12–14 represent oligomeric blocks of (LC10[KC10]ₙ)L with n number of repeating units ranging from 1 to 4. The sensitivity of ESI-ToF-MS decreases significantly with increasing n. Therefore, no direct conclusion can be drawn from the peak areas in the chromatograms. The products generated at different stages of the enzymatic degradation (moments in time) indicate that ester hydrolysis has dominated during enzymatic degradation up to day 18; after that, an unexpected change was observed and α-CT acted mainly as a protease cleaving predominately the amide bonds.

Figure 4 Selected TIC chromatograms of the degradation products obtained at different time intervals during α-chymotrypsin degradation at 37°C. Val and Phe represent valine and phenylalanine, respectively.

In case of PK, the appearance of L (peak number 3), C6 (4), benzyl alcohol (6), LC10K (7), LC10 (9), LC10KC10 (12), and so on indicated the cleavage of both ester and amide bonds. The enzymes in both cases showed preference towards the amide bond between L and C10 compared with the one between K and C10 because no release of K was observed (Figure 5). Table 2 shows the structural assignments of different peaks separated in Figures 4 and 5 based on the m/z ratios from their ESI-ToF mass spectra.
Chapter 3

Figure 5 Selected TIC chromatograms of the degradation products obtained after different time intervals during enzymatic degradation of the PEA with proteinase K at 37°C and 120 rpm.

Figure 6 Cumulative increase (y-axis) in the concentration (µg.mL⁻¹) of 1,6-hexanediol (◊ and ♦ symbols), sebacic acid (○ and ● symbols) and benzyl alcohol (▲ and △ symbols) during the course of enzymatic degradation of the PEA with α-chymotrypsin (solid lines) and proteinase K (dashed lines). The concentration of benzyl alcohol was determined by LC with UV-absorbance detection at 254 nm.

Because C6 is connected on both sides with L through ester bonds and C10 is bonded via amide bonds either to two K or to one L and one K, the emerging C6 and C10 can be used to monitor the ultimate degradation due to ester hydrolysis or amide bond-cleavage. Figure 6 demonstrates a nearly linear increase in the cumulative concentration of benzyl alcohol, C6
and C10 in the enzyme media collected after every 48 h during the enzymatic degradation with α-CT and PK. It is obvious from the quantitative data that the release of C10 greatly increased after the 18th day in α-CT degraded hydrolysates, whereas in case of PK, very little C10 was liberated. An interesting observation is that in case of the PK-mediated degradation the detected C6 in solution corresponds to 100% of the content of this moiety in the hydrolyzed polymer (estimation is based on the observed weight loss). However, more than half of the potential C6 appeared in fragments other than C6 itself when using α-CT. Although it is clear (cf. peaks nos. 3, 7 and 9) that PK exhibits amidase activity, the amount of C10 was negligible in the medium. This stresses the need for quantitative analysis of the intermediate degradation products.

Unfortunately, we can only analyze the oligomers in a semi-quantitative manner so far. The contribution of K, L, valine (Val) and phenylalanine (Phe) from the enzymes to the reaction media was confirmed by LC-ToF-MS analysis of the sample blank used in the study and also by the hydrolytic degradation of the enzymes.

3.5 Factors affecting enzyme activities

The effects of equilibrium or saturation of the degradation products on the activity of the enzymes were estimated by analyzing the reaction media incubated at 37°C and 120 rpm after 8, 24, 32, and 48 h by LC-ToF-MS. Figure 7 shows the peak areas for peaks from 11 to 15. In case of α-CT, the heights of these peaks are almost approaching almost constant levels. However, the peaks heights at 48 h are decreasing gradually as the molecular weights of the oligomers are increasing. This suggests further degradation of the water-soluble oligomers in the enzyme media at these prolonged incubating times. In case of PK, this situation has not yet been reached after 48 h.

The decrease in enzyme activity could be caused either by self-digestion or by denaturation of the enzyme. A gel-electrophoresis experiment was performed to compare a freshly prepared enzyme solution with a solution which had been stored at 37°C for 3 days [23]. This experiment clearly showed several small enzyme fragments in the stored sample.

The change in the activity of the α-CT during the enzymatic degradation was measured after 6, 18, 24, 30, and 48 h. At 0 h, the activity measurement involved only enzyme solution and the standard substrate (N-Suc-Ala-Ala-Pro-Phe-pNA). But after 6, 18, 24, 30, and 48 h, the enzyme media collected from the incubated coated disks also contained water-soluble degradation products from PEA. These products in the presence of standard substrate do not
appear to inhibit the activity of enzyme. Nevertheless, we found a decrease of >90% in the activity of enzyme after 48 h under the current experimental conditions.

Figure 7 TIC LC-ToF-MS chromatograms of the reaction media injected after 8 (Δ), 24 (□), 32 (○), and 48 (◊) hrs of enzymatic degradation. (a) α-chymotrypsin (b) proteinase K.

In summary, the PEA degraded primarily via ester hydrolysis during in vitro enzyme-mediated degradation (with α-CT and PK at 37°C). The survival of KC10-containing fragments under the applied enzymatic conditions suggested that the amide bonds between K and C10 are more stable than those between L and C10. The analysis shows only fragments, which theoretically could be derived directly from the anticipated polymer structure. This suggests a polymerization process without side reactions. A significant acceleration of the degradation may possibly be obtained if the enzyme solution is more frequently or even continuously refreshed. An on-stream-analysis system to study and explore these synergistic
effects is currently being developed in our laboratory. The rate-determining factor should still be the accessible surface area of the biomaterial, but the conditions will be more favourable for degradation than those used here.

**Table 2** Structural assignments for the fragments arising from different monomers and oligomers, separated as shown in Figures 4 and 5 following the enzyme-mediated degradation of the PEA in α-Chymotrypsin and Proteinase K. The symbols K, L, C6 and C10 represent lysine, leucine, 1,6-hexanediol, and sebacic acid, respectively.

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4 Conclusions

Poly(ester amide) polymer was subjected to enzymatic degradation conditions. Hyphenation of liquid chromatography with electrospray – time-of-flight – mass spectrometry proved to be a powerful analytical tool for the chromatographic separation and identification of the water-soluble degradation products after enzymatic degradation. The technique allowed the identification of fully and partially degraded polymer fragments, thus providing information on the polymerization process and on the intrinsic polymer structure. The polymer was found to degrade at a steady rate with both enzymes during this study. A lack of significant changes in the average molecular weight of the remaining polymer strongly suggests that surface erosion occurred during the enzyme-mediated degradation. Furthermore, no accumulation of acidic byproducts was observed during the course of the experiment. In this respect, the polymer performs better than conventional polyesters. The experiments confirmed that this class of (polyester amide)s shows a remarkable hydrolytic stability in the absence of enzymes.

Moreover, to avoid the further degradation of the water-soluble degradation products in the enzyme media due to long incubation time and to enhance the degradation rate by continuously refreshing the enzyme solution, the development of a system for on-stream analysis of degradation products is in progress.


5 References

6 Supporting information

Two-dimensional H,C-correlated NMR spectrum of the starting PEA. Mass spectra of the peaks identified during enzymatic degradation of PEA with α-CT and PK. Details of LC-ToF-MS method optimization for the separation and identification of the water soluble degradation products generated by means of chemical degradation of PEA. This material is available free of charge via the Internet at http://pubs.acs.org/.

6.1 Two-dimensional H,C-correlated spectrum (HSQC) of PEA

![Figure S1](image)

Figure S1 Two-dimensional H,C-correlated spectrum (HSQC) of the starting PEA, in d<sub>6</sub>-ethanol recorded on a Bruker Avance 400 MHz NMR spectrometer. Symbol ‡ are assigned to NH groups of amide bonds. The numbering to each signal corresponds to the numbering of protons and carbon present in Figure 1 in the Chapter 3.

6.2 ESI-ToF-MS spectra of the identified peaks – enzymatic degradation

The chromatographic peaks for the respective monomers and oligomers in Figure 4 and 5 were identified by their ESI-ToF-MS spectra (Table 2). Figure S2-S5 show the m/z values.
All the spectra showed protonated adducts. Sodium adducts were also observed due to the presence of sodium ions in the incubating buffer.

Figure S2 The LC-ToF-MS spectra of peaks 1, 3, 4, 5, and 7 in positive mode. The spectrum for peak 8 was recorded in the negative mode.
Monitoring the in vitro enzyme-mediated degradation of degradable PEA by LC-ToF-MS

Figure S3 The LC-ToF-MS spectra of peaks nos. 9-13 in positive mode.
Figure S4 The LC-ToF-MS spectra of peaks nos. 14-17 in positive mode.
6.3 Chemical Degradation – optimization of the LC-ToF-MS method

6.3.1 Chemical degradation

Approximately 20 mg of PEA were hydrolyzed at 120°C and 300 kPa pressure for 24 h while stirring in a 10 mL glass vessels with 2 mL of a mixture of 1 M NaOH and ethanol (75:25) in a microwave instrument (CEM Corporation, Methews, NC, USA)\textsuperscript{33}. Similarly, approximately 20 mg of PEA were mixed with 2 mL of a mixture of 3 M HCl and ethanol (75:25) and hydrolyzed at 90°C and 300 kPa for 24 h in the same apparatus as described above. Both hydrolysates were twofold diluted with LC-grade water and neutralized (pH ≈ 7) with HCl or NaOH prior to their injection into the LC-ToF-MS. Similarly, PEA in both mixtures were also hydrolyzed at room temperature.
6.3.2 Chemical degradation: LC-ToF-MS analysis

Figure S6a displays the TIC chromatograms for the partially hydrolyzed products in 1 M aqueous NaOH solution at room temperature. The peaks are labeled according the structural assignments based on their ESI-ToF mass spectra (Table S1). The formation of leucine (L) and 1,6-hexanediol (C6) indicates that the polymer is degraded primarily via ester hydrolysis. The amide bond between L and sebacic acid (C10) appears to be more susceptible to cleavage than the amide bonds between lysine (K) and C10. Peaks from 20.2 to 22.5 min represent LC10L and oligomeric blocks of (LC10(KC10)nL) with the number of repeating units (n) ranging from 1 to 5 in order of increasing retention. Because ethanol was added to enhance the solubility of the polymer in 1 M NaOH, ethylation may occur. This was observed for various peaks. For example, peak EtLC10K is the ethylated product of peak LC10K. Peaks from 22.5 to 26 min indicate the presence of ethylated products of peaks LC10, LC10KC10, LC10L, and oligomeric series LC10(KC10)1-3 (eluting in reverse order, as hydrophobicity increases upon ethylation). The ESI-ToF-MS spectra of peaks from 22.5 to 26 min show the co-elution of several singly ethylated products originating from LC10, LC10KC10, and LC10(KC10)1-3L (Table S1).

The polymer degraded nearly completely to its monomers in 1 M NaOH at 120°C in the microwave instrument (peaks 1, 3, 4 and 9) except for four fragments, namely C10K, LC10K, LC10, and LC10L. No ethylated products were observed (Figure S6b).

In case of acid hydrolysis (3 M HCl, at room temperature) the oligomers were separated according to the degree of ethylation i.e. without ethylation, once ethylated and twice ethylated oligomers, and once ethylated oligomers containing C6 at the other side (Figure S7a). Peaks EtLC10KEt, EtLC10(KC10)2Let, and EtLC10(KC10)1LEt reflect twice ethylated oligomers originating from the compounds associated with peaks LC10K, LC10(KC10)2L, and LC10(KC10)1L, respectively. Peaks EtLC10(KC10)2LC6, EtLC10(KC10)1LC6, and EtLC10LC6 contain additionally one ethyl group and C6 in comparison with the analytes of peaks LC10(KC10)2L, LC10(KC10)1L, and LC10L, respectively. Thus, the degradation products form a complex mixture of oligomers possessing different end-group functionalities (amine, alcohol, and carboxylic acid or ethyl esters). Based on hydrophobicity each class is eluted in a retention order with specific retention increments.
Monitoring the in vitro enzyme-mediated degradation of degradable PEA by LC-ToF-MS

Figure S6 TIC chromatograms of the PEA degraded in 1 M NaOH aqueous solution at (a) room temperature and (b) 120°C and 3 bar in a Microwave instrument.

Figure S7b shows the TIC chromatogram of the polymer degradation products after nearly complete degradation in 3M HCl:ethanol (75:25) at 90°C. At high temperature and under acidic conditions, esterification of the carboxylic-acid groups in L and C10 was observed (peaks EtL and EtC10). However, only one carboxylic acid was esterified in case of C10. Similarly, only one hydroxyl group of the C6 was etherified with ethanol to generate an ether group (peak EtC6). It has been reported in literature that the esterification of carboxylic acid with alcohols to produce esters is very slow at room temperature, but that direct microwave heating of the materials greatly speeds up the reaction [Pipus, G.; Plazl, I.; Koloini, T. Chemical Engineering Journal 2000, 76, (3), 239-245]. Lysine remained un-retained in all the chromatographic separations under the applied conditions and no esterification of its carboxylic-acid group was observed. The analysis shows only fragments which theoretically could be derived from the polymer structure, which suggests a polymerization process without side-reactions.
Figure S7 TIC chromatograms of the PEA degraded in 3 M HCl aqueous solution at (a) room temperature and (b) 90°C and 3 bar in a Microwave instrument.