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On-line microfluidic immobilized-enzyme reactors: A new tool for characterizing synthetic polymers

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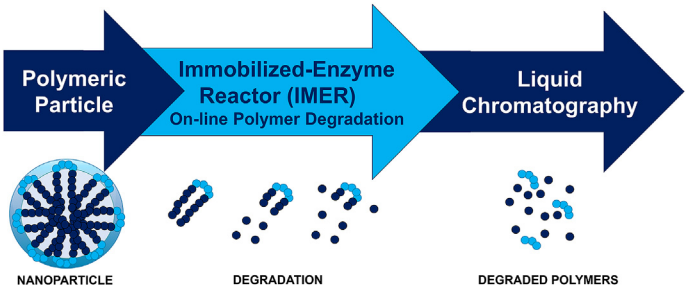
HIGHLIGHTS

- A microfluidic immobilized-enzyme reactor for polymer degradation was developed.
- Lipase was immobilized in a microfluidic reactor through vinylazlactone chemistry.
- The reactor was used for on-line degradation of polymeric nanoparticles.
- Degradation products were analysed on-line by size-exclusion chromatography.
- A residence time of a few minutes proved sufficient for full degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Biodegradable polymeric materials may eventually replace biostable materials for medical applications, including therapeutic devices, scaffolds for tissue engineering, and drug-delivery vehicles. To further develop such materials, a more fundamental understanding is necessary to correlate parameters including chemical-composition distribution within a macromolecular structure with the final properties of the material, including particle-size. A wide variety of analytical techniques have been applied for the characterization of polymer materials, including hyphenated techniques such as comprehensive two-dimensional liquid chromatography (LC × LC). In this context, we have investigated enzymatic degradation of polyester-based nanoparticles, both in-solution and by the use of an immobilized-enzyme reactor (IMER). We have demonstrated for the first time the implementation of such an IMER in a size-exclusion chromatography system for on-line degradation and subsequent analysis of the polymer degradation products. The effect of residence times ranging from 12 s to 4 min on polymer degradation was assessed. IMER-assisted degradation is much faster compared to in-solution degradation, which requires several hours to days, and opens the possibility to use such reactors in LC × LC modulation interfaces.

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1. Introduction

A biodegradable polymer can be defined as a polymer that is "susceptible to degradation by biological activity, with the degradation accompanied by a lowering of its molar mass" [1]. A distinction can be made between natural and synthetic biodegradable polymers, with cellulose and poly(glycolic acid) as examples of the respective categories. Biodegradable polymers may eventually replace biostable materials for medical applications, including their use as therapeutic devices, three-dimensional porous structures as scaffolds for tissue engineering, and drug-delivery vehicles [2]. One of the earliest examples and the most-extensively investigated class of biodegradable polymers is that of aliphatic polyesters, which are thermoplastic polymers with hydrolytically labile aliphatic-ester linkages in their backbone. Poly(glycolic acid), for example, has been used since the late 1960s as suture in medical applications [3]. To overcome disadvantages, such as rapid degradation and the accompanying formation of acidic products, poly(lactid acid) has been explored as a more slowly degrading polymer. In order to further tailor the properties of biodegradable polyesters to their application, multiple strategies have been proposed including *i*) the use of copolymers, such as poly(lactide-*co*-glycolide) (PLGA) polymers, the rate of degradation of which was found to depend on the lactic acid/glycolic acid ratio, monomer sequence, molecular weight, object shape, *etc.* [4,5], and *ii*) modifications of aliphatic polyesters with amides, anhydrides, urethanes, imides, or ethers [6]. As an example of the latter, polyethylene glycol (PEG) has been incorporated as a hydrophilic building block in PLGA-PEG-PLGA triblock copolymers to improve their biocompatibility [7].

Biodegradable polymers are complex macromolecules featuring a plethora of what Giddings has defined as sample dimensions [8]. These can include particle-size distribution, surface composition and charge, molecular weight, chemical-composition distribution and topology of the constituting molecules. While we are able to create virtually any type of polymer imaginable, a more fundamental understanding is necessary to correlate parameters such as chemical-composition distribution within a macromolecular structure with the final properties of the material. A wide variety of analytical techniques have been applied for the characterization of polymers and polymeric nanoparticles, including spectroscopic techniques (Raman and Fourier-transform infrared spectroscopy, FTIR, nuclear-magnetic-resonance spectroscopy, NMR), mass-spectrometric techniques (matrix-assisted laser-desorption/ionization mass spectrometry, MALDI-MS), pyrolysis gas chromatography–mass spectrometry (py-GC-MS), microscopy (scanning electron microscopy, SEM, atomic-force microscopy, AFM, transmission-electron microscopy, TEM), chromatography (size-exclusion chromatography, SEC, hydrodynamic chromatography, HDC, liquid chromatography, LC) and physical studies (differential scanning calorimetry, DSC, dynamic mechanical analysis, DMA, thermogravimetric analysis, TGA). As various properties of polymeric products are often interdependent, *e.g.* molecular weight and particle size, hyphenated analytical techniques, such as LC-NMR, LC-IR, LC-Raman, LC-MS, LC-MALDI, and LC-Py-GC, lead to valuable insights.

A technique which has been successfully applied for the separation of complex mixtures is comprehensive two-dimensional liquid chromatography (LC × LC). In the on-line, real-time version of LC × LC, a modulator is used to couple two separation dimensions with different time scales (spanning about two orders of magnitude) [9]. Selection of two sufficiently different ("orthogonal") separation mechanisms may yield separation according to two targeted properties of the sample mixture (*i.e.* sample dimensions) [8]. However, practical implementation of the technique is often hindered by a lack of (solvent) compatibility between the two intended separation mechanisms [10]. While active-modulation approaches have been developed to circumvent such issues [11,12], the separation of nanoparticles faces an additional challenge as the characterization of a number of properties requires analysis of the constituting polymers (*e.g.* polymer molecular weight, polymer composition, core-to-shell ratio, active-ingredient concentration). We recently combined HDC with SEC to simultaneously separate the particles based on their size and the polymers comprising the particles based on their molecular weight [13]. An on-line dissolution interface was developed, in which the separated nanoparticles were transformed into polymers. Using stationary-phase-assisted modulation, the dissolved analyte plugs were concentrated prior to injection into the second dimension. However, while successful in relating the particle-size distribution to the polymer molecular-weight distribution, the developed method cannot be applied to map the chemical-composition distribution. Indeed, to study such a property one would require (enzymatic) degradation of the polymers rather than chemical dissolution. In literature, many examples of enzymatic degradation of polymers can be found. An early example by Suzuki and co-workers reported the use of *Rh. delemar* lipase for the degradation of various aliphatic polyesters including poly (ethylene adipate) [14]. In a number of follow-up studies, polymer properties such as *i*) first-order structure (chemical structure, molecular weight [15]), *ii*) higher-order structure (glass-transition temperature, crystallinity [16]), and *iii*) surface conditions (surface area, hydrophilic and hydrophobic properties) [17] have all been found to affect the speed and degree of degradation.

In the context of proteomics studies, immobilized-enzyme reactors (IMER) have been applied for the (on-line) digestion of proteins. Through the immobilization of proteases such as trypsin, digestion times were reduced to seconds or minutes [18]. IMERs are more efficient for protein digestion than in-solution enzymatic digestion, because *i*) mass transfer between enzymes and substrate molecules occurs faster as the diffusion distances between the molecules are reduced and *ii*) the increased enzyme concentration in the reactor, which is only possible because autodigestion of the enzymes is prevented by their immobilization and, thus, isolation from each other. Additionally, an IMER allows (expensive) enzymes to be reused and the product stream is not contaminated by them. A study by Ghaffar et al. concerned online enzymatic degradation of polymers in an alternative setup [19]. The inside of a tube was coated with poly(ester amide)s, and an α-chymotrypsin solution was pumped through. Under different flow conditions, degradation products were collected and studied by reversed-phase liquid chromatography coupled with electrospray-ionizaton time-of-flight MS (RPLC-ESI-TOF-MS). However, to the best of our knowledge, an immobilized-enzyme reactor has never been applied for the (online) degradation of polymers.

In this contribution, a cyclic-olefin-copolymer microfluidic reactor has been constructed, containing lipase immobilized on a polymer monolithic material through a 2-vinyl-4,4-dimethylazlactone linker. We compared in-solution and IMER-facilitated enzymatic degradation of model copolymer nanoparticles consisting of poly(D,L-lactide-*co*-glycolide)-block-poly(ethyleneglycol)-block-poly(D,L-lactide-*co*-glycolide) (PLGA-PEG-PLGA, see Fig. S1 in Supplementary material section S-1), prior to size-exclusion chromatography hyphenated with evaporative light-scattering (ELS) detection and we assessed the effect of IMER residence time on degradation.

2. Experimental

2.1. Chemicals

All water referred to in this article was deionised (Arium 611UV; Satorius, Germany; resistivity = 18.2 MΩ. cm). Unstabilized tetrahydrofuran (THF) was obtained from Biosolve (Valkenswaard, The Netherlands). Poly(D,L-lactide-*co*-glycolide)-block-poly(-ethyleneglycol)-block-poly(D,L-lactide-*co*-glycolide) copolymer [PLGA-PEG-PLGA triblocks, number-average molecular weight, M_n = 6000-12000, PEG block M_n = 400, feed ratio lactide:glycolide 1:0.8], 4,4' bis(diethylamino) benzophenone (DEBP), ethylene glycol diacrylate, methyl methacrylate, 2,2-dimethoxy-2-phenylacetophenone, butyl methacrylate, ethylene glycol dimethacrylate, Al_2O_3, 1,4-butanediol, poly(ethylene glycol) methacrylate, tert-butanol, lipase from *Pseudomonas Sp.* (≥1200 U/mg), NaCl, ethanolamine, trypsin from bovine pancreas, and proteinase K from *Tritirachium album* were obtained from Sigma Aldrich (Steinheim, Germany). Acetone was obtained from Klinipath (VWR, Duiven, The Netherlands). Polystyrene and poly(methyl methacrylate) standards for construction of the calibration curves were obtained from Polymer Laboratories (now Agilent Technologies, Church Stretton, Shropshire, UK). 1-propanol was obtained from Acros (Geel, Belgium) and 2-vinyl-4,4-dimethylazlactone was obtained from Pure Chemistry Scientific (Watertown, MA, United States of America).

2.2. Instrumental

For size-exclusion chromatography (SEC), the setup comprised an Agilent 1100 series isocratic pump (G1310A), a 1290 Infinity autosampler (G4226A), a 1290 Infinity column compartment (G1316C) (Agilent, Waldbronn, Germany) in which an Agilent PLGel Mixed-E (300 × 7.5 mm i.d., 3-μm particles) SEC column was installed, and an Infinity 1260 Evaporative Light-Scattering detector (ELS, G4260B) (Agilent, Waldbronn, Germany).

For all on-line IMER × SEC-ELS experiments, the setup consisted of an Agilent 1100 series capillary μ-Pump (G1376A) in which a flow cell (G1376-60001) was installed with a maximum flow rate of 20 μL min^{-1}, the 1290 Infinity autosampler (G4226A), an 1100 series column compartment equipped with the IMER and a 1-μL heater (G1316-80012), which heated the mobile phase to 50 °C before it entered the IMER, and a 1290 Infinity column compartment (G1316C) equipped with an 8-port 2-position 2D-LC valve (5067-4214) with two 40-μL loops (5067−5425) to fractionate the IMER effluent. For the separation and detection, the same column and ELS detector were used.

2.3. Procedures

2.3.1. Sample preparation

For creating the nanoparticle dispersion, a 30,000-ppm solution of PLGA-PEG-PLGA triblocks was prepared in acetone. From this triblock solution, 0.4 mL was aliquoted into 10.0 mL of water under gentle mixing, resulting in the formation of the nanoparticles by nanoprecipitation. Hydrodynamic chromatography analysis indicated that the particles had an average size of 130 nm (see Supplementary Material, section S-2 for the corresponding data). Based on previous information we expect the diameter of the through pores of the IMER-containing monoliths to be in the range of 0.8−1.5 μm [18]. For recording the calibration curves, 5000 ppm solutions of all standards were individually prepared in unstabilized THF and diluted to 1000 ppm, also in unstabilized THF, for analysis (see Supplementary Material, section S-3 for the calibration curves).

2.3.2. Prototyping of microfluidic devices

The microfluidic devices were designed using SOLIDWORKS (Dassault Systèmes SOLIDWORKS, Waltham, MA, USA) and the features were micromachined using a micromilling machine (Datron M10 Pro, Datron, Mühltal, Germany). As a substrate material, 1.1 mm thick cyclic-olefin copolymer (TOPAS, grade 8007) plates were purchased from Axxicon Moulds Eindhoven B.V. (Eindhoven, The Netherlands). The microfluidic devices measured 45 mm × 15 mm, featured a 40 mm long straight channel with a 400 × 400 μm cross-section, 1.6 mm internal diameter entrance and exit holes on the top of the device, and two 1.6 mm through holes to facilitate alignment of the top and bottom plates during the bonding procedure. After milling the microchannels were thoroughly cleaned with deionised water and isopropanol (IPA) and dried. The devices were thermally bonded using a procedure analogue to one reported previously [20]. For micro-to-macro connections, the devices were placed in a Fluidic Connect PRO chipholder frame (Micronit Microtechnologies BV, Enschede, The Netherlands).

2.3.3. Enzyme-immobilization process

The procedure to immobilize enzymes *in-situ* in the microfluidic channels was adapted from the method described elsewhere [18], and consisted of three major steps: *i*) surface modification of the cyclic-olefin copolymer microchannel and *in-situ* polymerization of the polymer monolith, *ii*) photografting of poly(ethylene glycol) methacrylate and 2-vinyl-4,4-dimethylazlactone, and *iii*) lipase immobilization. During the last step, a 2000 mg/L aqueous solution of lipase containing NaCl (0.9% by weight) was pumped through the microdevice for 2 h at 0.5 μL min^{-1}. Subsequently, an 1 M ethanolamine aqueous solution was pumped through the reactor for 1 h at 0.5 μL min^{-1} to quench unreacted azlactone groups. Finally, reactors were flushed with and stored in NaCl aqueous solution (0.9% by weight) at 4 °C until use.

The ratio between monomers (*i.e.* BuMA and EDMA) and porogens (*i.e.* 1,4-butanediol and 1-propanol) in the polymerization mixture was 40:60. Assuming a 96% complete conversion of monomers to polymers [21], the final porosity for the polymer-monolithic structure inside the channel is 62.5%. As the dimensions of the microfluidic channel are 40 × 0.4 × 0.4 mm ($L \times w \times h$), the IMER has a void volume of approximately 4 μL.

2.3.4. Analytical methods

2.3.4.1. In-solution degradation. For the in-solution-degradation experiments, 0.5 mL of the nanoparticle solution was mixed with 0.5 mL of a 2000-ppm solution of the enzyme of interest in water. The actual enzyme concentration during in-solution degradation was, therefore, 1000 ppm. The resulting solution was then placed in an oven at 37 °C (although the optimal temperature for lipase has been reported to be 50 °C [16]). 20 μL were sampled for SEC analysis after 0, 0.5, 1, 1.5, 2, 24, and 118 h of degradation.

The SEC analysis was conducted at a flow rate of 1.5 mL·min^{-1}and a temperature of 60 °C (see section 2.2 for setup). The analysis time was 7.0 min. The ELS detector was set to a nebulizer temperature of 80 °C, an evaporation temperature of 65 °C and a nitrogen gas flow rate of 1.6 L/min. Because of the possible formation of peroxides, unstabilized THF was not used beyond two to three days after opening a fresh bottle, for all SEC analyses discussed in this entire study.

2.3.4.2. On-line IMER degradation. For all on-line IMER-degradation experiments described in sections 3.3 and 3.4 the following method parameters were used. The injection volume of the autosampler into the IMER was 20 μL. The flow rate in the IMER dimension for results shown in section 3.3 was 1 μL min^{-1}, whereas

a range of flow rates (1, 2, 4, 8, 12, 16, 20 μL min^{-1}) were used for the experiments shown in section 3.4. The temperature in the column compartment containing the IMER and preheater was set to 50 °C. The column compartment holding the 2D-LC valve and SEC column was maintaining a temperature of 60 °C.

Taking into account that, as a result of the Poiseuille flow-profile, a 20-μL sample plug requires a loop of roughly double the volume [12], and considering the volumes of the various components of the setup, it was calculated and verified that the partially degraded nanoparticle plug leaving the IMER would occupy the 40-μL loop at time point 28.5 min when the flow rate in the IMER dimension was 1 μL min^{-1}. During experiments conducted at this flow rate, the 2D-LC valve was switched so as to comprehensively inject the entire nanoparticle plug into the SEC column for conducting the SEC separation. For experiments operated at higher flow rates (section 3.4) the valve-switching time was reduced accordingly. The SEC method itself operated as described in section 2.3.3.1.

2.4. Data analysis

To construct molecular-weight-distribution (MWD) plots from obtained SEC data, a calibration curve was recorded using poly(-methyl methacrylate) (PMMA) and polystyrene (PS) standards (See Supplementary Material section S3 for a full explanation and the calibration curve).

3. Results & discussion

3.1. In-solution degradation

Known for their ability to hydrolyse ester bonds, trypsin and lipase were selected as potential candidate enzymes for immobilization in a microfluidic reactor for the degradation of the aliphatic polyester PLGA-PEG-PLGA. Trypsin has been reported for the degradation of polyesters [22] and it has been immobilized in a microfluidic reactor for the degradation of proteins in an earlier study [18]. Lipase was included for similar reasons as it has been investigated for the degradation of polymers and polyester nanoparticles [23].

To compare the performance of different enzymes, in-solution enzymatic-degradation experiments were conducted prior to immobilization. PLGA-PEG-PLGA nanoparticles were formed by nanoprecipitation, mixed with the enzymes, and samples were taken over time and analyzed using SEC-ELS (see section 2.3.3). Fig. 1 displays the molecular-weight-distribution plots, which were constructed from the chromatograms (see 2.4) for lipase (A), trypsin (B), and a control sample without enzyme (C). As time progressed, smaller polymers were observed in the distribution indicating polymer degradation. While it is true that the intensity of the polymer distribution was not constant, no shift in the median molecular weight could be observed towards smaller molecules for the control sample. As a result, there was no evidence of polymer degradation and a more plausible reason for the irregularity in the distribution intensity would be inhomogeneity of the sample or variations in the injection or sampling process. Most importantly, neither enzyme was found to be capable of fully digesting the polymers within 2 h in-solution.

In Fig. 2, the median molecular weight is plotted against degradation time. For both enzymes, a clear shift in the median molecular weight is visible after 24 h, yet significant differences in enzymatic activity can be observed. Interestingly, the activity of lipase was found to be higher than that of trypsin in the first 3 h, despite the sub-optimal operating temperature of 37 °C for lipase (below the reported optimal temperature of 50 °C [16]). Although the data in Fig. 2A suggests a more complete degradation of the polyesters using trypsin after a number of days, the action of this enzyme is too slow for it to be used in an enzyme-assisted modulation interface in LC × LC. Fig. 2 furthermore illustrates that the PLGA-PEG-PLGA triblocks do not degrade as a result of acid- or base-catalyzed ester hydrolysis in the aqueous medium in the absence of enzymatic activity.

It should be noted that a multitude of different types of lipase and trypsin enzymes exist, with different optimal conditions in terms of pH and temperature. Additionally, the optimal conditions for free and immobilized enzymes can be different. This was particularly important, because almost all of the above parameters could also affect the stability of the nanoparticles, solubility of polymers or their degradation products, and the integrity of the monolithic stationary phase. The faster polymer degradation by lipase in the first 3 h under sub-optimal temperature conditions prompted us to continue with this enzyme for immobilization in a microreactor.

3.2. On-line degradation of PLGA-PEG-PLGA by IMER

The construction of a cyclic-olefin-copolymer-based microfluidic reactor and enzyme immobilization within the microfluidic channel have been previously described in a publication regarding trypsin-assisted protein digestion [18] and has been adapted here for lipase-immobilization. Briefly, cyclic-olefin copolymer was selected as a substrate material for prototyping, mainly due to its excellent chemical resistance to solvents typically used for liquid chromatography and its transparency in the near-ultraviolet and visible light ranges, which is essential for photo-polymerization and -grafting of functional groups. When implemented within LC × LC modulation interfaces, the microfluidic reactor may be exposed to pressure drops of up to 20 MPa. As described elsewhere [24], micromachined layers of cyclic-olefin copolymer were bonded using a solvent-vapour-assisted bonding method, which has resulted in average burst pressures of 38 MPa. Finally, the immobilized-enzyme reactor (IMER) was placed in an aluminium holder in a temperature-controlled compartment of the 2D-LC instrument, connecting the device with NanoPort connections to various hardware modules. The inlet of the IMER was coupled to the autosampler through a preheating channel inside the column compartment. As enzyme activity is known to be influenced by temperature, the preheater was used to introduce the mobile phase and triblock polymers at the appropriate temperature, optimal for the enzyme. The outlet of the IMER was coupled to a 2D-LC switching valve, which transferred the IMER effluent to the SEC separation (as illustrated in Fig. 3).

In SEC, separation is based on partial exclusion of molecules from the stationary phase [25,26]. Molecules which are too large to penetrate the pores, and thus are excluded, travel faster through the column than molecules which permeate into the pores, where the effective flow rate is much lower. As a result, very small molecules, such as sample-solvent molecules, will generally elute around the dead time of the column. Molecules that are too large to enter the pores generally elute near the exclusion limit, which is typically around half the dead time. Consequently, our SEC chromatograms are plotted on the τ-scale, where τ is the ratio between analyte elution time, t_e, and the column dead time or hold-up time, t_0, *i.e.* $\tau = t_e/t_0$, and $\tau = 1$ corresponds to t_0.

To study the ability of the IMER to digest the PLGA-PEG-PLGA nanoparticles, two different devices were tested initially. For the first device, the whole procedure described in section 2.3.3 was carried out resulting in a device with immobilized lipase. For the second device, the lipase-immobilization step was omitted from the procedure, so that it could be used as a control. Based on the

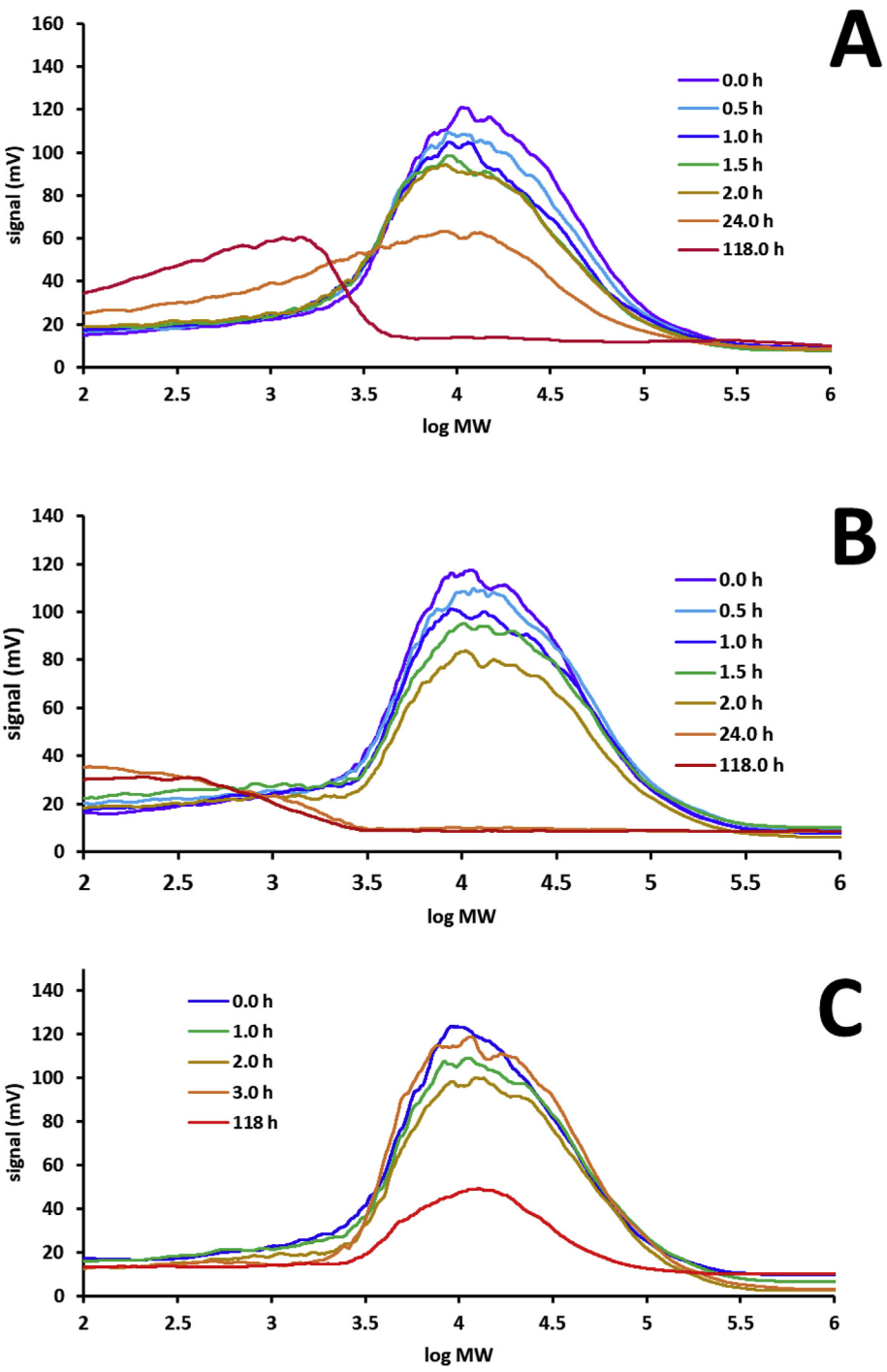

Fig. 1. In-solution degradation of PLGA-PEG triblock copolymers after formation of micelles by nanoprecipitation, with lipase (A), trypsin (B), and without enzyme addition as a control (C). Samples were analyzed by SEC-ELS after 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 24 h, and 118 h degradation.

dimensions of the microfluidic reactor (see section 2.3.2) and a monolith porosity of $\varepsilon = 0.625$, the dead volume of the reactor was estimated to be 4 μL. For both chromatograms shown in Fig. 4, the residence time was therefore 4 min at a flow rate of 1 μL min^{-1}. The blue, solid line shown in Fig. 4 is the SEC chromatogram recorded using a device without immobilized lipase. A distribution of larger molecules can be observed in this chromatogram. Conversely, this distribution is missing and a much larger signal around t_0 is observed in the pink, dashed SEC chromatogram which was recorded using a device with immobilized lipase. Consequently, it can be concluded that the only difference between the two devices, the presence or absence of lipase, is responsible for polymer degradation. The overlay clearly shows the presence of polymers in the effluent from the device without immobilized lipase, whereas high-molecular-weight analytes were not observed in the effluent from the device with immobilized lipase.

The results of this experiment *i*) proved that lipase was still enzymatically active, despite its covalent attachment to the polymer-monolith surface, *ii*) supported the earlier observation that non-enzymatic degradation of the polymer due to ester

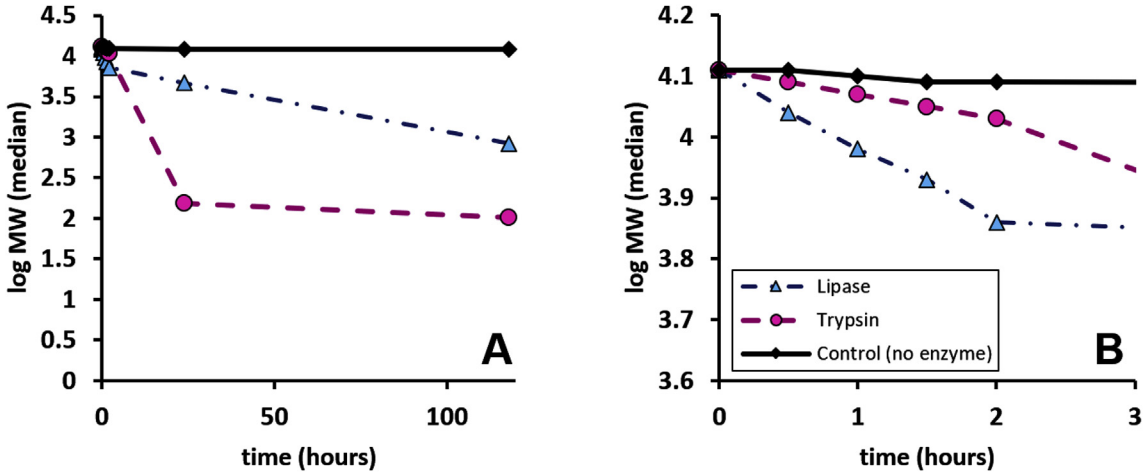

Fig. 2. Median molecular weight plotted against degradation time, with lipase (triangular symbols, blue) or trypsin (circular symbols, pink) added and without enzyme added as a control (diamond-shaped symbols, black). B shows the first 3 h of degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

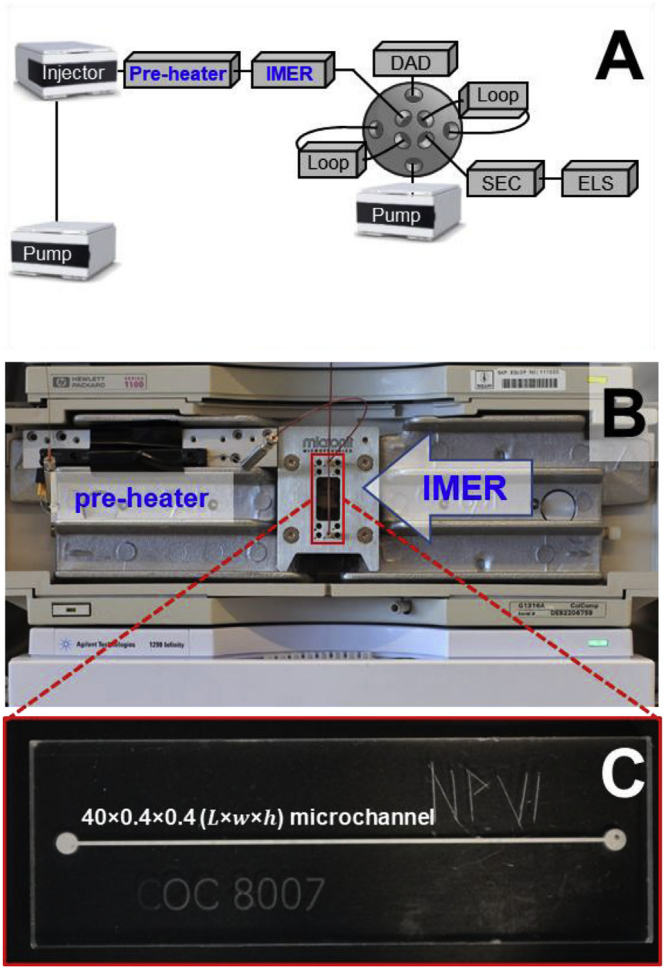

Fig. 3. (A) Schematic overview of the setup used for online IMER-facilitated polymer degradation and analysis of the degradation products. (B). Microfluidic IMER placed in its aluminium holder in a temperature-controlled compartment, connecting the device with NanoPort connections to the autosampler and switching valve. (C) Close-up photograph of the microfluidic IMER.

hydrolysis by the aqueous mobile phase was not significant under these conditions and, most importantly, *iii*) demonstrated that the IMER was able to fully degrade the PLGA-PEG-PLA triblock nanoparticles into smaller fragments within the residence time of 4 min, whereas in-solution degradation required many hours for only partial degradation. However, the chromatograms in Fig. 4 give an incomplete impression of the IMER effluent. The data does provide insight in whether polymers are still present after elution from the IMER, but no information is obtained on the resulting degradation products. These are of high interest, but subsequent on-line analysis is not straightforward. The degradation of PLGA-PEG triblocks results in highly hydrophilic lactic-acid and glycolic-acid products and PEG chains, which rules out the possibility of using reversed-phase LC. The 100% aqueous medium also rules out hydrophilic-interaction chromatography (HILIC) as the effluent would have to be diluted with an enormous excess of acetonitrile.

3.3. *Influence of residence time on polymer degradation*

The reactor residence time represents the time available for the enzymes to degrade the polymer. This relates to the modulation time if the reactor were implemented in a comprehensive 2D-LC (LC × LC) system. A short modulation time is crucial to prevent undersampling of the first-dimension effluent, yet full degradation of the polymer must be accomplished. To investigate this trade-off, a nanoparticle solution was injected and subjected to IMER degradation using different flow rates to achieve a range of residence times. The results are shown in Fig. 5.

Fig. 5A shows an overlay of SEC chromatograms for the investigated flow rates. To display the very low molecular weight signal ($\tau > 0.8$), beyond the covered range of the calibration curve ($0.45 < \tau < 0.8$), the curves were plotted on the τ-scale. The number between brackets in the legend reflects the approximate residence time in min. As expected, a longer residence time results in more-complete degradation. A residence time of 2 min suffices for the polymer to be completely degraded. This is at the high end of the range of typical modulation times in used in LC × LC.

Future studies will thus aim to apply the present technology in an LC × LC flow stream. In addition, additional enzymes may be studied, such as proteinase K. Typical LC × LC methods make use of organic solvents, salts, and surfactants as mobile-phase additives, which may have a significant impact on the performance of the immobilized enzymes. Proteinase K has been observed to display

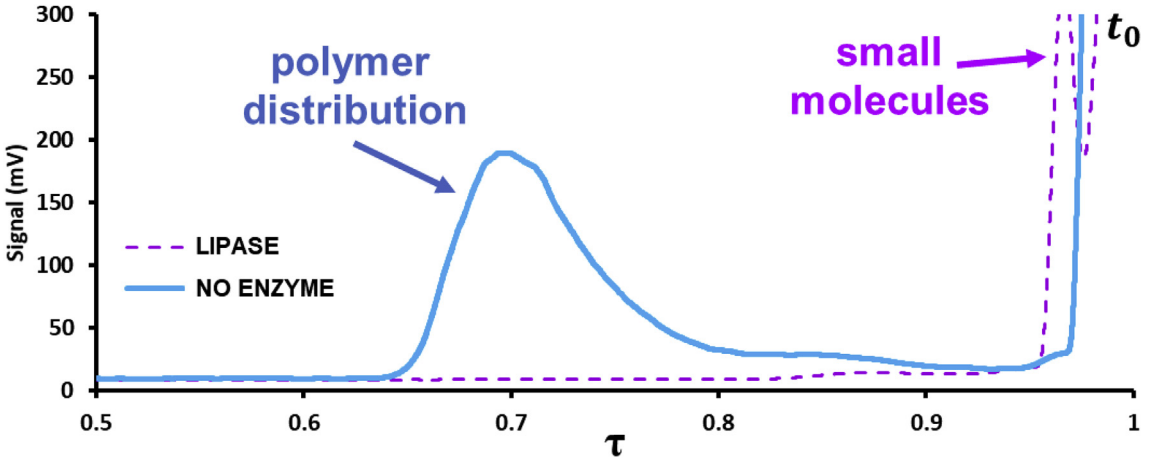

Fig. 4. Overlay of SEC-ELS chromatograms of device effluent after injection of PLGA-PEG-PLGA nanoparticles into devices containing a monolithic surface with (dashed, pink line) and without (solid, blue line) immobilized lipase. Using the immobilized-lipase reactor, no large polymers were observed, and the peak around t_0 ($\tau = 1$), corresponding to small molecules, had intensified. Note that τ is the ratio between the analyte elution time, t_e, and the column dead time or hold-up time, t_0, *i.e.* $\tau = t_e/t_0$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

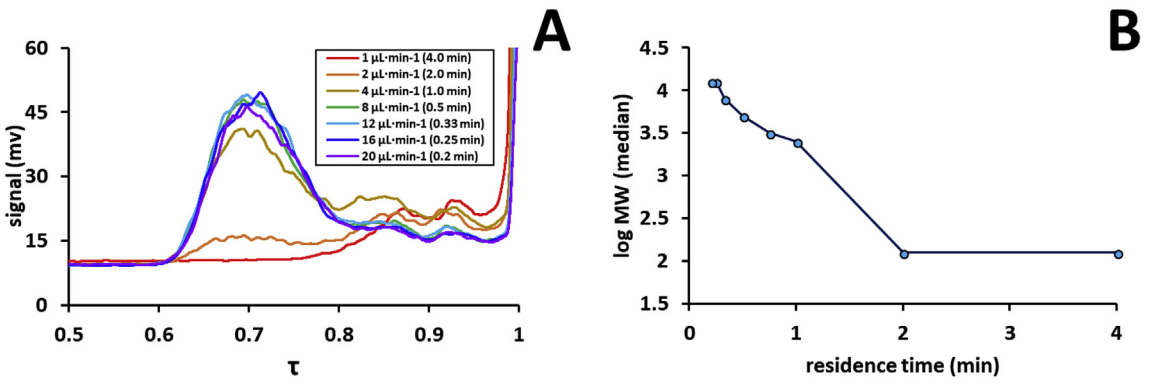

Fig. 5. Effect of residence time on PLGA-PEG triblock degradation by use of the immobilized-lipase reactor. A range of reactor residence times from 12 s to 4 min was assessed by varying the flow rate through the reactor with a volume of about 4 µL. SEC-ELS chromatograms are shown in (A) with the legend reflecting the approximate residence time (min) in brackets, while (B) shows the relation between the reactor residence time and the median molecular weight of the polymers. Note that τ is the ratio between analyte elution time, t_e, and the column dead time or hold-up time, t_0, *i.e.* $\tau = t_e/t_0$, and $\tau = 1$ corresponds to t_0.

an increased enzyme activity in interaction with specific organic solvents [27].

Moreover, it is important to note that the poly(BuMA-*co*-EDMA) monolithic support in the IMER also comprises ester bonds in its backbone and on its surface. It is, therefore, possible that these ester bonds are also hydrolysed by the enzyme, but only during the enzyme-immobilization step of the reactor-preparation procedure by free "in-solution" enzymes. Once immobilized, the enzymes are suspended above the monolith surface by a dipeptide spacer molecule and can only interact with the molecules they encounter through diffusion/convection. We have not studied the extent of degradation, if any, during the immobilization step.

4. Conclusion & outlook

We have demonstrated for the first time the successful development and operation of an immobilized-enzyme reactor capable of degrading PLGA-PEG-PLGA nanoparticles within a residence time of 2 min. This entails a massive improvement in comparison with in-solution degradation, which requires several hours to days and hence opens the possibility to use such reactors in LC × LC modulation interfaces. We aim to implement the present reactors in a novel type of reaction modulator, which will serve as an interface in a multi-dimensional liquid chromatography system to enable on-line enzymatic degradation of biodegradable polymers during sample transfer. Insight into various parameters such as chemical-composition distribution and functionality-type distribution may be obtained by studying degradation products in a second dimension.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2018.12.002.

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