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Fast *in vitro* hydrolytic degradation of polyester urethane acrylate biomaterials: Structure elucidation, separation and quantification of degradation products

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ABSTRACT

Synthetic biomaterials have evoked extensive interest for applications in the field of health care. Prior to administration to the body a quantitative study is necessary to evaluate their composition. An *in vitro* method was developed for the quick hydrolytic degradation of poly-2-hydroxyethyl methacrylate (pHEMA), poly(lactide-co-glycolide50/50)1550-diol (PLGA(50:50)$_{1550}$-diol), PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ containing ethyl ester lysine diisocyanate (etLDI) linkers using a microwave instrument. Hydrolysis time and temperature were optimized while monitoring the degree of hydrolysis by ^{1}H NMR spectroscopy. Complete hydrolytic degradation was achieved at 120 °C and 3 bar pressure after 24 h. Chemical structure elucidations of the degradation products were carried out using ^{1}H and ^{13}C NMR spectroscopy. The molecular weight (MW) of the polymethacrylic backbone was estimated via size-exclusion chromatography coupled to refractive index detection (SEC-dRI). A bimodal MW distribution was found experimentally, also in the pHEMA starting material. The number average molecular weights (M_n) of the PLGA-links (PLGA(50:50)$_{1550}$-diol) were calculated by high pressure liquid chromatography–time-of-flight mass spectrometry (HPLC–TOF-MS) and ^{1}H NMR. The amounts of the high and low MW degradation products were determined by SEC-dRI and, HPLC–TOF-MS, respectively. The main hydrolysis products poly (methacrylic acid) (PMAA), ethylene glycol (EG), diethylene glycol (DEG), lactic acid (LA), glycolic acid (GA) and lysine were recovered almost quantitatively. The current method leads to the complete hydrolytic degradation of these materials and will be helpful to study the degradation behavior of these novel cross-linked polymeric biomaterials.

1. Introduction

Synthetic polymeric biomaterials are of high importance in the medical field due to an aging population and their potential to improve the quality of life [1]. There is a gradual trend to replace non-degradable materials with degradable materials mainly because of the need to avoid reinterventions when complications arise with non-degradable materials [2]. This is most vividly seen with the move in the stent coating area where stable drug eluting coatings are being replaced with biodegradable coatings [3]. Such kinds of materials have their potential use as joint and limb replacements [4], artificial arteries [5] and skin [6], contact lenses [7], dental implants [8], catheters [9], in tissue engineering [10] and as systems for controlled delivery of drugs [11], etc. An important class of degradable biomaterials are chemically cross-linked polymeric networks predominantly based on pHEMA and PLGA [12,13]. Since its birth in 1936 [14] and first reported application for contact lenses in 1960 [15], pHEMA is one of the most extensively studied polymeric biomaterials in biomedical applications [16] because of its biocompatibility, hydrophilicity, softness, high water content and permeability [17], but it has poor mechanical properties [18]. However, numerous studies reported the modification of the hydroxyl group with poly(ε-caprolactone) (PCL) [3], poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [19], dextran [11], poly(2-(dimethylamino)ethyl methacrylate) [20], poly(ethylene oxide) [12], poly(tetrahydrofurfuryl methacrylate) [21], poly(ethylene

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glycol)-methacrylate [22], poly(dimethylsiloxane) [23], sulfopropyl methacrylate [24] and cross-linker to tune the biomechanical properties of the pHEMA.

PLGA is an FDA-approved biodegradable and biocompatible polymeric biomaterial [25]. PLGA is widely used as a drug delivery matrix using numerous forms such as microspheres [26], nanoparticles [27], scaffold [28], microfibers [29], tablets [30], in the field of control release delivery devices, and tissue engineering. Currently, the focus on synthesis of copolymers of PLGA with other polymers has been increased such as PLGA-PCL-PLGA [31], MeO-PEG-PLGA-PEG-OMe [32], PLGA-PEG [33] and PLGA-grafted dextran [34].

Chemical and enzymatic hydrolysis are the primary biodegradation mechanisms for such materials. Phagocyte-derived oxidants, produced as a result of foreign body response, may also contribute to the *in vivo* degradation of aliphatic ether groups in these networks [35]. The suitability of the polymeric biomaterials for medical devices can be inferred from their chemical structure, the degradation time and the biocompatibility of the polymers and their degradation products [11]. Swelling ratios (water contents) of the hydrogels [10,12], weight loss [10,23], pH of the medium [36], kinetic chain length [37], etc. are the most common parameters used to assess the *in vitro* degradation of material. These parameters may be insensitive in the early stages of degradation and are not very informative on toxicology. Chromatographic methods that can give more insight into the structure of these networks and can be used to predict their properties more accurately are desired. However, networks lack solubility, a prerequisite for such analysis. This requires a very sensitive method of analysis, or at least an accelerated *in vitro* chemical hydrolysis of the novel biomaterials at extreme pHs or high temperature, possibly avoiding the formation of any insoluble product, followed by the structural analysis and quantification of their degradation products. The collected information will be helpful not only (i) to ascertain the composition of the original networks, but also (ii) to evaluate the biocompatibility of these polymeric networks and their degradation products and (iii) to modify the existing and to design new biomaterials for specific applications. Recently, Matsubara et al. reported a supercritical methanolysis to achieve the selective decomposition at ester linkages in a UV-cured acrylic ester resin to characterize the cross-linking structures, but no quantification of the decomposition products was done to assess the degree of methanolysis [38].

A more detailed second approach to study these prospective biomaterials is a chemical or a specific enzymatic degradation during physiological conditions, allowing one to study the kinetics of degradation. Again, specific and sensitive chromatographic methods will be needed to draw sound conclusions. In particular a method is needed as the second stage in a two-step procedure and is reported here. First degradation under physiologically relevant conditions is performed, resulting in partially degraded material of which the constituents may be identified. Then complete and fast degradation of the products of the first step (oligomers, intermediates and other products) is executed for quantification.

In the present study polymeric biomaterials based on pHEMA (backbone) and PLGA(50:50)$_{1550}$-diol (PLGA-links) were subjected to fast hydrolytic degradation. One reason to select these samples is that pHEMA, frequently formed as an intermediate hydrolysis product in polymeric network biomaterials is only partially hydrolyzed under physiologically relevant conditions [11] and no detailed study on the complete hydrolytic degradation and direct analysis of its degradation products has yet been published to our knowledge.

In this paper first the development and optimization of a method for the microwave-assisted *in vitro* hydrolytic degradation is reported of pHEMA, PLGA(50:50)$_{1550}$-diol and the photo-crosslinked polymeric biomaterials such as PLGA(50:50)$_{1550}$(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$. The hydrolysis of polymeric biomaterials and their model building blocks, pHEMA (backbone) and PLGA(50:50)$_{1550}$-diol (PLGA-links) were performed at up to 120 °C, for different periods of time. The hydrolysis time and the temperature were optimized while monitoring the degree of hydrolysis in the starting material with ^{1}H NMR spectroscopy. Then the structure elucidations of the degradation products (Fig. 1) were carried out using ^{1}H and ^{13}C NMR spectroscopy and quantification of high MW hydrolyzed polymethacrylic acid backbone by SEC-dRI and LA, GA, EG, DEG, and lysine by HPLC–TOF-MS in the hydrolyzed sample are reported. The MW distribution of the hydrolyzed backbone was estimated via SEC-dRI. The Mn of the PLGA-links was measured by HPLC–TOF-MS and ^{1}H NMR.

2. Experimental

2.1. Materials

DL-Lactide and glycolide were purchased from PURAC (CSM Biochemicals, Gorinchem, The Netherlands), ethyl ester of lysine diisocyanate from Kyowa Hakko Europe GmbH Dusseldorf, Germany), caprolactone from Solvay, methacryloyl chloride via Fluka. Irganox 1035 was obtained from Ciba-Geigy (Basel, Switzerland). pHEMA [M_v = 300 kDa (192,066) or 20 kDa (529,265), solvent and temperature conditions of M_v determination are not known] and all other chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA). The chemicals were used as such unless otherwise stated. All water used was deionized.

The experimental batches of PLGA(50:50)$_{1550}$-diol, PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ were synthesized at DSM Biomedical, Geleen, The Netherlands, according to the following procedure:

Preparation of PLGA(50:50)$_{1550}$-diol: DL-lactide (51.6 g, 0.358 mol), glycolide (41.5 g, 0.358 mmol) and diethyleneglycol (6.85 g, 6.45 mmol) were weighed in the glovebox and melted at 150 °C under nitrogen conditions. 1 mL of a stock solution Tin(II)-ethylhexanoate (290 mg in 10 mL *n*-hexane) was added as a catalyst. The reaction was allowed to proceed for 18 h upon which the reaction mixture was cooled to room temperature to obtain poly(lactide-co-glycolide50/50)1550-diol [39].

Preparation of PLGA(50:50)$_{1550}$-diol(HEMA)$_2$: poly(lactide-co-glycolide50/50)1550-diol (100 g, 65 mmol), 200 mg Irganox 1035 and triethyl amine (13.05 g, 0.129 mol) were dissolved in 150 mL dry tetrahydrofuran. Methacryloylchloride (13.49 g, 0.129 mol) was added drop wise to the solution at controlled temperature (<5 °C). Immediately a white precipitate was visible (triethylamine.HCl salt). The dropping funnel was rinsed with THF (50 mL). The reaction mixture was stirred at room temperature for 18 h. The reaction mixture cooled till 5 °C and filtered to remove the triethylamine.HCl salt. The THF was removed via evaporation with a rotavapor. The remainder was dissolved in 200 mL ethyl acetate. The clear solution was extracted once with 300 mL 0.1 HCl solution, once with 300 mL 5% NaCl-solution and 300 mL water. The resulting solution was dried with $NaSO_4$ and evaporated to dryness. poly(lactide-co-glycolide50/50)1550-dimethacrylate was obtained as a slightly coloured yellow oil. 30.49 g poly(lactide-co-glycolide50/50)1550-dimethacrylate, 13.1 g HEMA and 0.86 g Darocur 1173 was mixed in a clear formulation [39].

Microparticles preparation of PLGA(50:50)$_{1550}$-diol(HEMA)$_2$: 10.52 g of this formulation was mixed with 39.88 g PEG 35k (40% m/m in water), 30.0 g water and 5 g aceton. This mixture was stirred mechanically for 10 min at 800 rpm before polymerization. The polymerization was allowed to proceed for 60 min under UV light (Macam Flexicure controller, D-bulb, 200 mW/s/cm^2, Livingston, United Kingdom). After polymerization, the micro particles

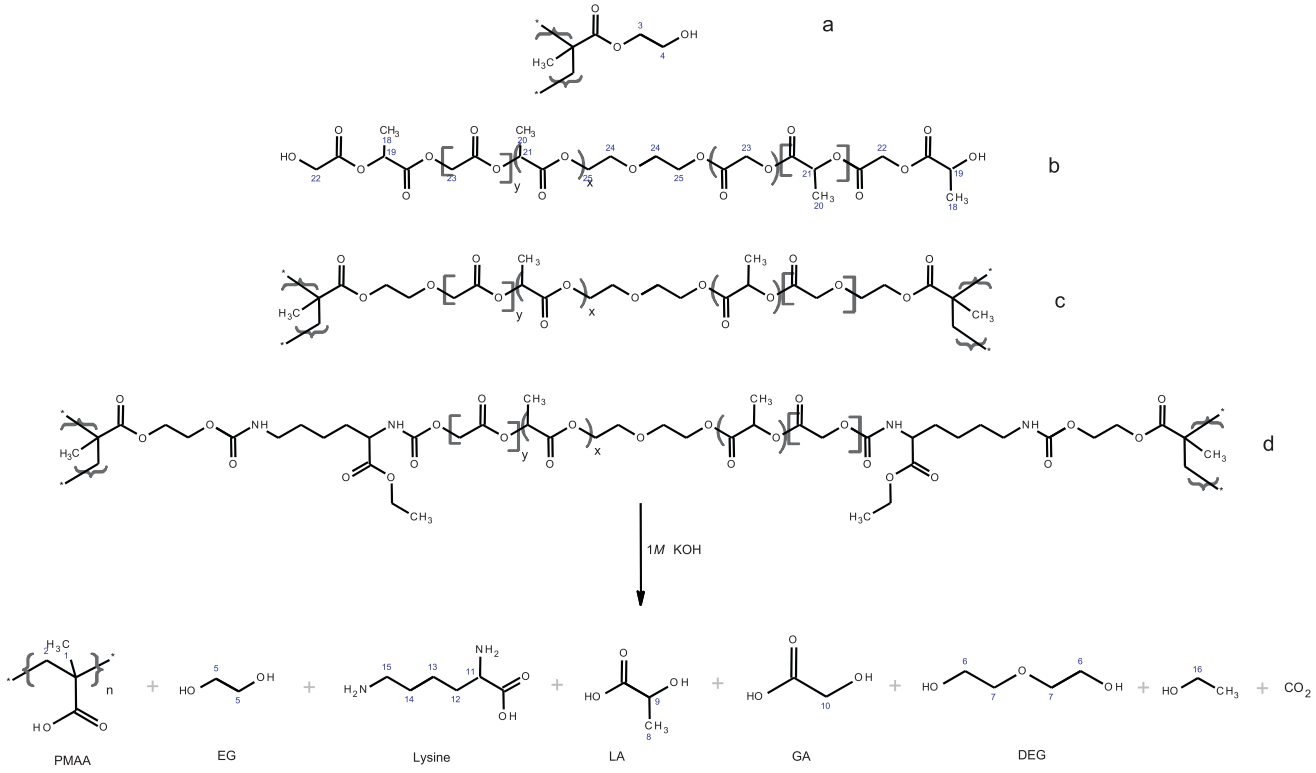

Fig. 1. Proposed reaction scheme for the hydrolytic degradation of (a) pHEMA, (b) PLGA(50:50)$_{1550}$-diol, (c) PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and (d) PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$. PMAA represents poly(methacrylic acid); EG, ethylene glycol; DEG, diethylene glycol; LA, lactic acid and GA, glycolic acid. The numbering corresponds to NMR peak assignments in Fig. 6.

were filtered through a 0.8 μm filter (Supor-800, Gelman Sciences, Ann Arbor, MI, USA) under vacuum and rinsed with 250 mL water. The morphology was checked with light microscopy. The methacrylate conversion was >96%. The micro particles were sieved afterwards using ethanol as solvent (Retsch sieves, aperture 63, 125 and 250 μm, Haan, Germany). The microparticles were dried via freeze drying [40].

Preparation of PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$: Hydroxymethylacrylate (HEMA, 26.0 g, 0.20 mol) was added drop wise to a solution of the ethyllester of Lysinediisocyanate (etLDI) (45.25 g, 0.2 mol), Tin-(II)-ethylhexanote (0.080 g, 0.186 mmol), Irganox 1035 (0.260 g) and dry air at controlled temperature (<5 °C). Subsequently the reaction mixture was stirred overnight at 40 °C. The etLDI-HEMA was obtained as a slightly yellow oil. The reaction was monitored with GPC w.r.t. to the presence of HEA (no HEMA visible). poly(lactide-co-glycolide50/50)1550-diol (100 g, 0.064 mmol) was dissolved in 150 mL dry THF. etLDI-HEMA (46.05, 0.129 mol) was added to the reaction mixture at room temperature. Subsequently the reaction mixture was stirred overnight at 40 °C. In the morning the reaction mixture was analysed with IR (no NCO peak $\nu = 2260\ cm^{-1}$ visible). When the reaction was complete, based on IR spectroscopy the THF was evaporated. The poly(lactide-co-glycolide50/50)1550-(etLDI-HEMA)$_2$ was obtained as a yellowish oil [40].

Microparticles preparation of PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$: 15.58 g PLGA$_{1550}$-diol(etLDI-HEMA)$_2$ and 0.31 g Darocure 1173 where mixed together mechanically at 100 RPM in a 250 mL beaker at 50 °C, now 62 g PEG35K (40.0% *m*/*m* in deionized water) and 58 g deionized water where added. This was stirred mechanically for 30 min at 900 rpm. The polymerization was allowed to proceed for 60 min at 70 °C 900 rpm under UV light (Macam Flexicure controller, D-bulb, 200 mW/s/cm^2). The particles were wet-sieved with deionized water over a sieving tower (Retsch test sieve Aperture 250, 125, 63 and 45 μm) and dried under vacuum at room temperature for 18 h. Afterwards methacrylate conversion was checked: >98% (FT-IR, 1640 cm^{-1} and 815 cm^{-1}) [40].

2.2. Procedure of hydrolysis

20 or 40 mg of each sample was dissolved in 2 mL of 1 M KOH (Merck, Darmstadt, Germany) in a 10-mL pressurized glass vial (CEM Corporation, NC, USA) using a magnetic stirrer. The 10-mL pressurized glass vial (ID = 12 mm) was internally lined with a PTFE tube of 1 mm thickness and ID = 11 mm (locally made at the mechanical workshop of the University of Amsterdam, Fig. 2). The homogeneous mixture in the glass vessel was placed in the Microwave instrument (Discover *BenchMate*, CEM) and hydrolysis to PMAA and EG was carried out at 120 °C, 3 bar and for 24, 20, 15, 10 and 5 h. Similarly, PLGA(50:50)$_{1550}$-diol, PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ andPLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ were hydrolyzed at 120 °C for 24 h at 3 bars. The mixture was weighed before and after each hydrolysis.

2.3. ^{1}H NMR spectroscopy of hydrolysate

0.5 mL of each hydrolysis solution was acidified by adding carefully a few drops of 37% HCl with vigorous stirring at 90 °C. The PMAA precipitates and along with supernatant (containing ethylene glycol (EG), diethylene glycol (DEG), lactic acid (LA), glycolic acid (GA), lysine, etc.) were dried overnight at 40 °C with an air flush.

The dried mixtures of the hydrolysates were re-dissolved in d_4-methanol (Euriso-top, France). Samples of un-hydrolyzed pHEMA and PLGA(50:50)$_{1550}$-diol were also prepared in d_4-methanol. ^{1}H NMR spectra were recorded on a Varian Inova 500 MHz NMR (Varian Inc., USA) equipped with Probe: 500 5 mm 13C/31P/1H GS. Pulse repetition time: 25 s, Pulse: 3.6 μs, Scans: 63 and temperature: 25 °C were used to record ^{1}H spectra.

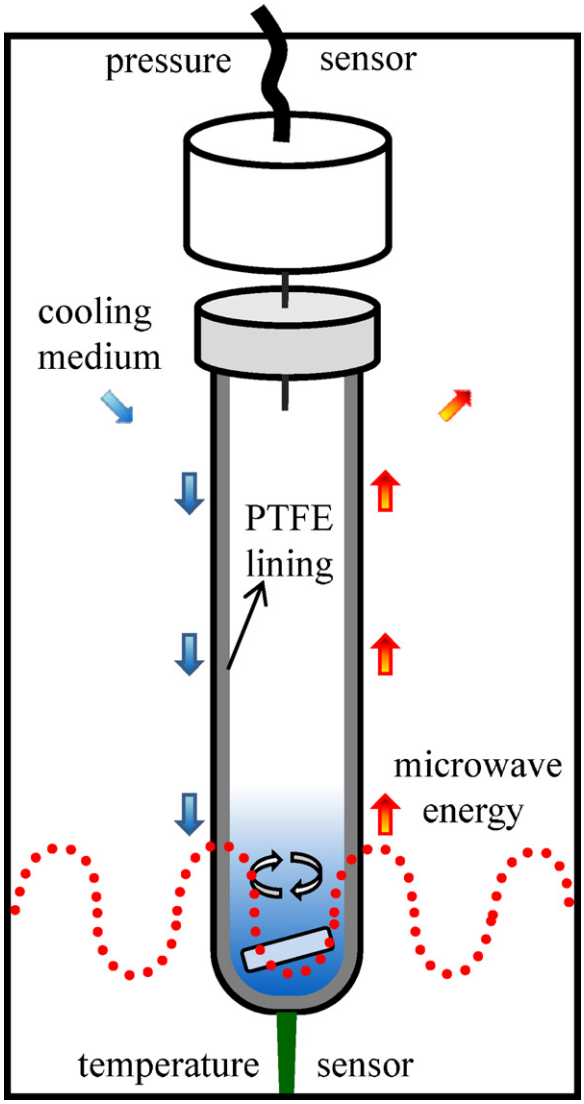

Fig. 2. Schematic diagram of CEM Discover microwave apparatus used in this work, with additional PTFE lining of 1 mm thickness.

2.4. Size exclusion chromatography (SEC) analysis

pH neutralized (0.2 mL) hydrolysis solutions were diluted with 0.2 mL aqueous SEC mobile phase. The SEC experiments were performed on an HPLC system equipped with in-line degasser, Model 600 pump, 717 plus TRI-SEC auto-sampler and Model 410 differential refractive index detector (all Waters, Milford, MA, USA). Data were recorded and chromatographic peaks were treated using Empower 2 software (Waters, Milford, MA, USA). Calculations for molar mass distribution (MMD) on the chromatographic peaks were executed using software written in-house in Excel 2003 (Microsoft).

All aqueous SEC separations were performed on the following set of columns used in series: PL Aquagel-OH Guard (8 μm, 50 mm × 7.5 mm), PL Aquagel-OH 50, 30 and 10 (each 8 μm, 300 mm × 7.5 mm) columns (Polymer Laboratories, UK). For 20 kDa pHEMA hydrolysates, same set of columns was used except PL Aquagel-OH 50. The mobile phase was (0.2 M $NaNO_3$, 0.01 M NaH_2PO_4, pH 7) pumped at a flow rate of 1.0 mL min^{-1}. Poly(methacrylic acid) sodium salt (PMA-Na) standards (Table 1) were used to calibrate the SEC-dRI system. The calibration curves for molar mass determination of PMAA in 300 and 20 kDa pHEMA hydrolysates are given by cubic relations of log M and retention time, x: $\log(M) = -0.00915 \times 3 + 0.51649 \times 2 - 9.98592x + 70.73912$, $R^2 = 0.999$ and $\log(M) = -0.032 \times^3 + 1.218 \times^2 - 15.62x + 73.11$, $R^2 = 0.998$, respectively. To quantify the concentration of hydrolyzed backbone as poly methacrylic acid sodium salt (PMA-Na) in hydrolysates the calibration lines were recorded using PMA-Na standards with Mp 65.8 kDa (at six concentrations 0.2–2 mg mL^{-1}) and 22.5 kDa (at five concentrations 1–5 mg mL^{-1}). Highly pure water for mobile phase preparation was obtained by means of an Arium® 611 Ultrapure (18.2 MΩ cm) Water System (Sartorius AG, Goettingen, Germany).

Table 1
Peak molecular weight (M_p), weight average molecular weight (M_w), number average molecular (M_n) and PDI of the poly(methacrylic acid) sodium salt standards. Data as specified by the supplier.

Standard	M_p (D)	M_w (D)	M_n (D)	PDI	Supplier
PMA-Na-1	1220	1250	1040	1.197	PSS
PMA-Na-2	1670	1700	1520	1.120	PSS
PMA-Na-3	3180	3150	2700	1.169	PSS
PMA-Na-4	7830	7750	7220	1.073	Fluka
PMA-Na-5	8210	8280	7480	1.108	PSS
PMA-Na-6	22,500	22,100	21,100	1.047	PSS
PMA-Na-7	31,500	31,100	30,400	1.023	Fluka
PMA-Na-8	65,800	62,500	60,600	1.031	PSS
PMA-Na-9	78,300	75,100	73,300	1.025	Fluka
PMA-Na-10	201,000	192,000	186,000	1.029	PSS
PMA-Na-11	480,000	421,000	380,000	1.108	PSS
PMA-Na-12	549,000	483,000	429,000	1.126	Fluka

Size-exclusion chromatography of pHEMA (300 and 20 kDa) was performed on two PL gel 5 μ MIXED-C (8 μm, 50 mm × 7.5 mm) columns with DMF (Acros Organics, NJ, USA) containing 0.02 M lithium chloride (Acros Organics) as a mobile phase pumped at a flow rate of 1 mL min^{-1} via an LC-10AD solvent delivery module coupled with a RID-10A dRI detector (Shimadzu Corporation, Kyoto, Japan). A Rheodyne 7120 manual injector (Rheodyne Europe GmbH, Alsbach, Germany) with 20 μl loop was used as an injection system.

The resolving power of a SEC system can be visualized by an integrity plot, which gives the integrity index (II_{sec}) as a function of sample M_w and ($M_w/M_n - 1$) [41]. (II_{sec}) indicates the fraction of dispersion of the experimental peak variance that is caused by the polydispersity of the sample itself and not by dispersion due to the column or extra-column band broadening [42]. The integrity plot for the used SEC system was constructed using the polymer standards listed in Table 1 and clearly demonstrates its suitability even for narrowly distributed polymers in the range of 2–200 kDa (c.f. Fig. 3).

2.5. HPLC–ESI-TOF-MS analysis of hydrolysate

0.1 mL of the hydrolysate was mixed with 4 mL of deionized (DI) water and then pH neutralized. The mixture was filtered with a 0.2 μm pore size PTFE filter (Grace Davison discovery science, IL, USA), prior to injection. Stock solutions of LA (Fluka), GA (Fluka), EG (Aldrich), DEG (Fluka) and D-lysine (Sigma) were prepared by dissolving in 1 M KOH solution and quantification was done with a standard addition method in order to correct for signal suppression of target analytes by co-eluting compounds. The chromatographic separations were performed on a Prevail C18 column (250 mm × 4.6 mm, 5 μm particle size, Alltech Discovery Sciences, IL, USA) at temperature of 35 °C. The injection system consisted of a Rheodyne 7010 manual injector (with 5 or 20 μL loops). The aqueous mobile phase containing 0.1% (v/v) formic acid (Fluka), 0.03% (w/v) NaI (Aldrich) and 1.0% (v/v) acetonitrile (Biosolve) was pumped via Shimadzu LC-20AD solvent delivery module at 2 mL min^{-1} and was split between the column and electrospray

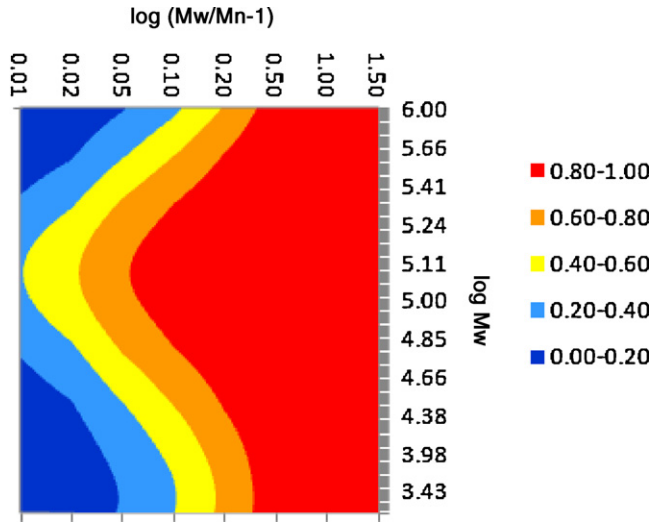

Fig. 3. Experimental SEC-integrity plot as a function of the sample (horizontal axis; $M_w/M_n - 1$) proportional to log(PDI-1)) and molecular weight (vertical axis; log M)). System: PL aquagel-OH Guard (8 μm, 50 mm × 7.5 mm), PL aquagel-OH 50, 30 and 10 (each 8 μm, 300 mm × 7.5 mm) columns; mobile phase: 0.2 M $NaNO_3$, 0.01 M NaH_2PO_4, pH 7) pumped at a flow rate of 1.0 mL min^{-1}.

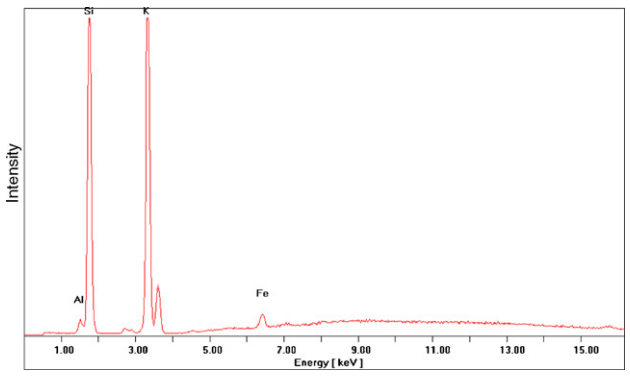

Fig. 4. XRF spectrum of white residues without lining the pressurized glass vessel with PTFE.

ionization (ESI) interface by means of a zero dead volume T-piece to assure a flow of approximately 0.2 mL min^{-1} into the ESI interface. Organic solvents used for the LC mobile phase were of HPLC grade.

In order to evaluate the recovery of the procedure of the method about 10 mg each of PMA-Na standard (M_p = 78.3 kDa), DEG, EG and D-lysine and approx. 50 mg of LA and GA standards were dissolved in 5 mL (5.2 g) of 1 M KOH to make control solutions. 2 mL of the mixture was heated at 120 °C and 3 bar in the microwave for 24 h. The concentration of each analyte was determined before and after heating to calculate the percentage recovery of the method for each analyte.

The LC system was hyphenated with an Agilent 6210 series Time-of-Flight Mass Spectrometer (Agilent Technologies, Waldbronn, Germany) via an ESI interface. The conditions of the ESI-TOF-MS were as follows: drying gas was nitrogen (N_2) at 8.0 L min^{-1}; and at 300 °C; 30 psig of N_2; capillary voltage, 3500 V; fragmenter, 140 V; skimmer voltage, 60 V; octopole dc1, 33 V; octopole radio frequency, 250 V. The data were acquired in the scan mode from m/z 50 to 500 D with 0.88 scans/s. An Agilent MassHunter Workstation A.02.01 and Analyst™ QS 1.1 software (Applied Biosystems) were used for data acquisition and data analysis, respectively.

3. Results and discussion

3.1. Optimization of hydrolysis method

Initially the hydrolysis method was optimized by degrading the pHEMA (300 and 20 kDa) in a 10-mL pressurized glass vial specially designed for the CEM microwave instrument. After hydrolysis the glass vessel contained the hydrolysis solution and white material, stuck on the wall of the glass vessel. We were not able to dissolve this material, for further analysis by NMR, SEC or HPLC, except at very low pH. The residues were originally considered to be silicates. For further analysis, these residues were washed three times with 5 mL of water, methanol and DMF to wash out possible impurities of hydrolyzed and un-hydrolyzed pHEMA and dried overnight at 210 °C in oven. The XRF spectrum (Eagle-III Spectrometer, EDAX Inc., Mahwah, NJ, USA) of these residues confirmed the presence of silicates primarily originating from the glass vessel in alkaline conditions at high temperature (Fig. 4). However, the CHN elemental analysis (Truspec, Leco, Germany) also revealed the presence of carbon contents in this material. Based on the percentage of these carbon contents, it can be concluded that up to 35% of the starting material (pHEMA) is lost by inclusion in the white residue from the hydrolysis solution. It may be assumed that at high temperature the highly reactive silanol groups present on silicates react with hydroxyl groups of pHEMA [43].

The formation of white residues during hydrolysis will lead to wrong quantification of the relative percentage of starting material in the hydrolysis solution, so to avoid contact of the alkaline solution with the inner surface of the glass vessel (to prevent the formation of white residues) it was internally lined with PTFE (Fig. 2). The hydrolysis was performed repeatedly after this modification and no formation of white residues was observed. The hydrolysis time was optimized while monitoring the cleavage of ester linkages in pHEMA with ^{1}H NMR spectroscopy. Then the hydrolysis of PLGA(50:50)$_{1550}$-diol, PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ was conducted at 120 °C for 24 h.

3.2. Product identification

The overlay of ^{1}H NMR spectra of pHEMA hydrolyzed for different times (Fig. 5) show clearly the cleavage of ester groups of pHEMA i.e. the scission of side chains from the backbone chain and the formation of free ethylene glycol (peak 5 at δ 3.6 ppm) and a small quantity of diethylene glycol (peak 7 at δ 3.56 ppm and peak 6 at δ 3.68 ppm). The peaks of free ethylene glycol and diethylene glycol were confirmed by taking the ^{1}H NMR spectrum of sample spiked with EG and DEG standards in CD_3OD. The signals at δ 3.75 ppm (peak 4) and δ 4.21 ppm (peak 3) corresponding to the side chain of pHEMA almost vanished after 24 h, indicating the degree of hydrolysis of pHEMA. In the starting material before hydrolysis the position of peaks 3 and 4 was observed at δ 4.08 and δ 3.80 ppm, respectively. DEG observed in the ^{1}H NMR spectra is present as an impurity in pHEMA.

The two-dimensional homonuclear (H,H)-correlated NMR experiment indicates the proton connectivity between signals at δ 3.80 and δ 4.08 ppm in the starting material and at δ 3.75 and δ 4.21 ppm (peaks 3 and 4) for the hydrolysate affirming the presence of the side chain in both the starting material and the hydrolysate. Also the proton connectivity in the signals (peaks 6 and 7) of diethylene glycol was confirmed. The ^{13}C NMR spectra in DEPT135 mode were recorded to assign the methyl, methane and methylene group and quaternary carbon in both the starting material and the hydrolyzed products. The single bond connectivities between ^{1}H and ^{13}C were also determined by the two-dimensional ^{13}C, ^{1}H-correlated HSQC NMR experiments.

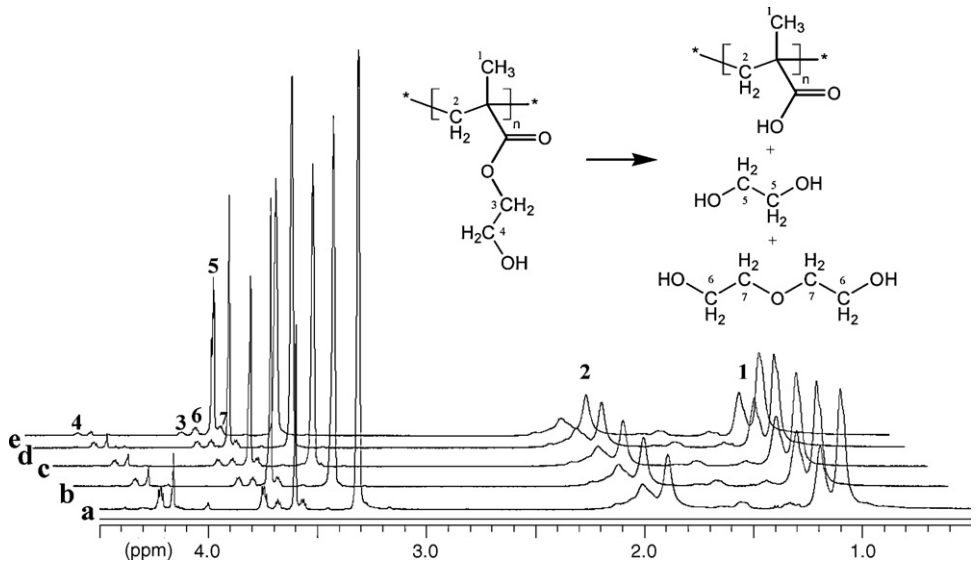

Fig. 5. An overlay of ^{1}H NMR spectra (CD_3OD, 25 °C, 500 MHz) of pHEMA (300 kDa) hydrolyzed after different times of hydrolytic degradation (a) 5 h, (b) 10 h, (c) 15 h, (d) 20 h and (e) 24 h at 120 °C in the microwave instrument.

The relative percentage of non-hydrolyzed pHEMA in the hydrolysis solution of pHEMA (300 kDa) and pHEMA (20 kDa) was determined using the following equation:

$$\text{Relative \% of pHEMA} = \frac{5 \times A_{3.7-4.3}}{4 \times A_{1.0-2.0}} \times 100 \quad (1)$$

In which A_x is the peak area for the response at the shift of x ppm.

The relative percentage of non-hydrolyzed pHEMA in the hydrolysate of 300 kDa pHEMA decreases from 6% at 5 h to less than 0.2% at 24 h. pHEMA with M_v = 20 kDa degraded much faster.

The ^{1}H NMR spectra of PLGA(50:50)$_{1550}$-diol before (Fig. 6A) and after hydrolysis (Fig. 6B) show the cleavage of ester bonds in PLGA(50:50)$_{1550}$-diol chains and the formation of free LA (CH_3 = 1.33 ppm and CH = 4.0 ppm), GA (CH_2 = 3.88 ppm) and DEG (HO–CH_2– = 3.7 ppm and –O–CH_2– = 3.58 ppm). More acidic hydroxyl groups are formed after hydrolysis and this leads to more hydrogen bonding. Consequently, the hydroxyl group signal shift towards higher frequency after hydrolysis. The multiple signals for the methylene groups (number 22) indicate sequential or tacticity effects (starting material is DL-lactide). Unfortunately, the signal at 1.15 ppm is an isopropanol impurity (an experimental artifact).

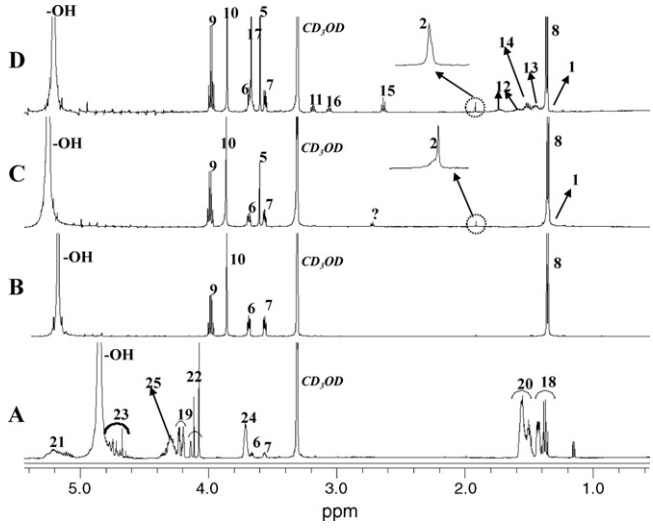

Fig. 6. The 1H-NMR spectrum of (A) PLGA(50:50)$_{1550}$-diol before hydrolysis and (B) PLGA(50:50)$_{1550}$-diol (C) PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ (D) PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ after hydrolysis at 120 °C for 24 h. The numbering of peaks corresponds to the numbering in Fig. 1.

The liquid NMR of PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ polymeric biomaterials were not possible because of their lack of solubility in any of the available NMR solvents. Upon hydrolysis it was possible to dissolve the acidified and non-acidified degraded backbone (PMAA/PMA-NA) in CD_3OD and D_2O, respectively. PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ is a 3D ladder like polymer network consisting of backbones of pHEMA interconnected via cross-links of PLGA(50:50)$_{1550}$-diol. If all the ester bonds are cleaved, the polymer network should result in polymethacrylate backbone, EG, DEG, LA and GA. The ^{1}H NMR of hydrolyzed PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ (Fig. 6C) shows that the CH_3– signal (peak 1) that belongs to the poly methacylate backone is overlapped by the CH_3– signal of LA (peak 8). This was confirmed by ^{1}H–^{1}H gCOSY (two-dimensional homonuclear H, H gradient-correlated spectroscopy) NMR experiments. The peak 2 belongs to the –CH_2– group of the backbone. Rest of the signals corresponds to the hydrolyzed PLGA(50:50)$_{1550}$-diol ^{1}H NMR spectrum (Fig. 6B). The peak that appears at 2.72 ppm is unknown.

The PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ is similar in chemical structure to PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ except that the backbones of pHEMA and PLGA-links (PLGA(50:50)$_{1550}$-diol) are interconnected via ethyl ester L-lysine diisocyanate (etLDI) linkers. So upon complete hydrolytic degradation all the products should be the same as in case of PLGA$_{1550}$-diol(HEMA)$_2$ except for lysine and ethanol. The signals 11, 12, 13, 14, 15 (Fig. 6D) belong to lysine (Fig. 1). The signal bond connectivity between lysine signals were established by ^{1}H–^{1}H-gCOSY. The peak 16, a triplet, belongs to a CH_2– group of ethanol. Peak 17 belongs to PEG present as an impurity in the biomaterial. M_n and the molar ratios of LA and GA in the PLGA(50:50)$_{1550}$-diol were calculated by integrating the CH group of LA, –CH_2– group of GA and –CH_2– groups of DEG (Table 2).

HPLC–TOF-MS is much more sensitive than NMR, but it is an indirect method: determination of LA and GA occurs after hydrolysis. Still, the M_n of the PLGA-links measured by HPLC–TOF-MS is considered to be more accurate, because in NMR accurate integration of the peak area is difficult as the signals of EG and DEG slightly overlap. This results in apparently higher M_n values by NMR except in case of PLGA(50:50)$_{1550}$-diol links (Table 2).

Table 2
M_n of PLGA-links (PLGA(50:50)$_{1550}$-diol) and molar ratios of LA and GA calculated by ^{1}H NMR and HPLC–TOF-MS.

Sample	^{1}H NMR			HPLC–TOF-MS		
	M_n (Da)	% RSD (n = 3)	Molar ratio (LA:GA)	M_n (Da)	% RSD (n = 3)	Molar ratio (LA:GA)
PLGA(50:50)$_{1550}$-diol	1317	5	51:49	1371	2	49:51
PLGA(50:50)$_{1550}$-diol(HEMA)$_2$	1549	2	50:50	1001	3	48:52
PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$	1791	2	50:50	1028	4	49:51

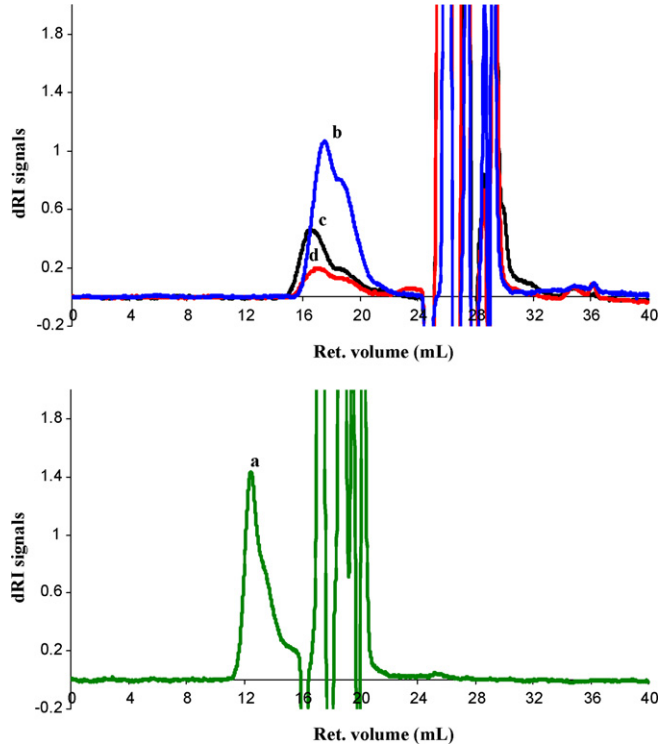

Fig. 7. SEC-dRI chromatograms of (a) pHEMA 20 kDa, (b) pHEMA 300 kDa, (c) PLGA(50:50)$_{1550}$-diol(HEMA)$_2$, and (d) PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ after hydrolysis for 24 h at 120 °C and 3 bars pressure.

3.3. Molar mass characterization and quantification of PMAA

Fig. 7 is showing the aqueous size exclusion chromatographic separation for the hydrolyzed backbone (in the form of PMA-Na) for the samples subjected to hydrolysis: pHEMA 20 kDa (a), pHEMA 300 kDa (b), PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ (c) and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ (d). All the peaks show a non-Gaussian distribution. One potential cause of this bimodal MMD is that two chains are cross-linked due the esterification reaction between alcohol in one chain and carboxylic acid in the other chain. There is no evidence of cross-linking found in ^{1}H NMR spectra, so the extent of cross-linking is very small and the signals may not appear in the spectra. Zainnuddin et al. suggested that the presence of ions in the solute can promote the formation of physical

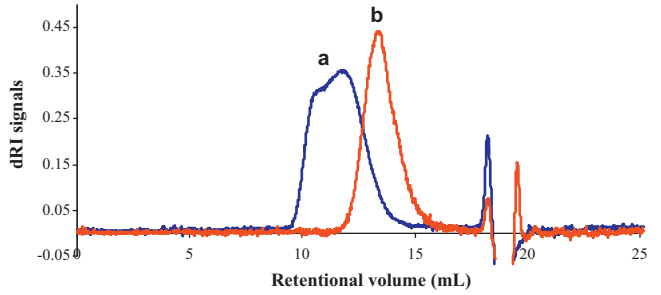

Fig. 8. SEC-dRI chromatograms of starting materials (a) 300 kDa and (b) 20 kDa pHEMA.

crosslinks between two neighboring hydrophilic and hydrophobic groups of the polymer chains [44]. However, it is unlikely that these physical cross-links stay intact when subjecting the sample to SEC.

In order to see the effect of hydrolysis on bimodality of the hydrolysate product, PMA-Na standard with Mp 549 kDa, which showed a uni-modal MMD without hydrolysis, was hydrolyzed in 1 M KOH with and without EG at 120 °C and 3.0 bar for 24 h. The hydrolysates were subjected to SEC and no influence of the hydrolysis was observed on the uni-modality of the MMD. The bimodal distribution of PMA-Na was independent of injection volume. pHEMA with low MW is soluble in water but as the MW increases, its solubility in water decreases [45]. Therefore, to further investigate the origin of bimodality in the hydrolyzed backbone, SEC in DMF of pHEMA (300 and 20 kDa) was performed. The MMD of 300 kDa pHEMA renders a non-Gaussian behavior (Fig. 8), so the bimodal distribution appears already present in the starting material.

In 20 kDa pHEMA hydrolysate the PMAA elutes from 11 mL to 16.2 mL and shows a small shoulder on the low molecular weight side. Fig. 7a indicates that very low MW (<1000 Da) PMAA is eluting together with the high concentration of salt. (This could be solved by application of columns with a resolving range at low molecular weights.) Therefore, the M_n value for 20 kDa pHEMA hydrolysate is not reported in Table 3. The M_n, M_w and PDI of PMA-Na in the samples are listed in Table 3. The hydrolyzed backbones in case of PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ are more polydisperse than those of the pHEMA (20 and 300 kDa) standards. The monomeric products of hydrolysis such as LA, GA, EG, DEG and lysine elute after the permeation limit. The quantitative results of hydrolyzed backbone in different biomaterials are tabulated in Table 5.

Table 3
SEC-dRI data for the hydrolyzed backbone (PMA-Na).

Sample	M_n (kDa)	% RSD (n = 3)	M_w (kDa)	% RSD (n = 3)	PDI	% RSD (n = 3)
300 kDa	52	8	108	6	2.1	7
20 kDa	–	–	16	1	–	–
PLGA(50:50)$_{1550}$-diol(HEMA)$_2$	46	4	223	3	4.7	4
PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$	34	8	152	6	4.5	9

The molecular weight data are PMA-Na-relative.

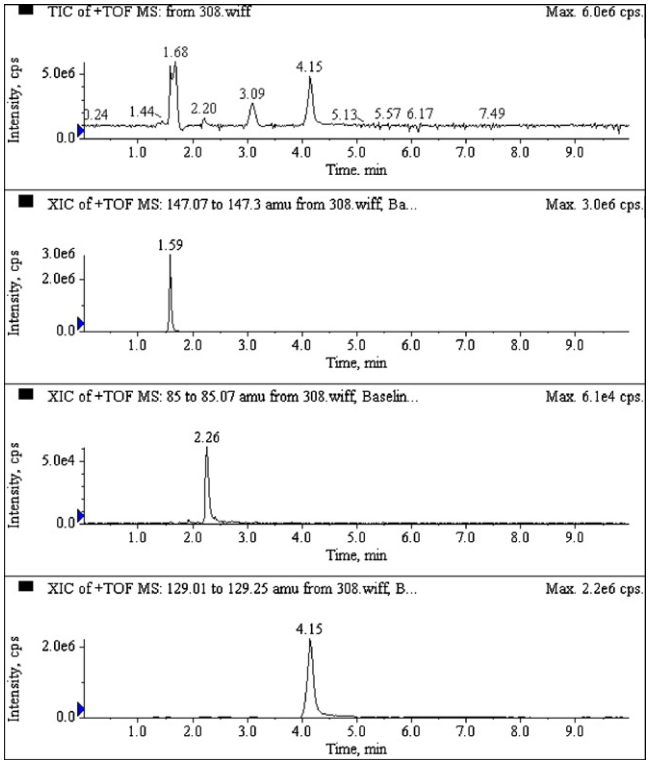

Fig. 9. TIC and XIC chromatogram of PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ hydrolysate. Conditions: positive ESI mode with isocratic elution with water containing 0.1% (v/v) formic acid, 0.03% (w/v) NaI and 1% (v/v) acetonitrile, flow rate 1.5 mL min^{-1}, column C18 Alltech Prevail (250 mm × 4.6 mm, 5 μm). Peaks at 1.59, 2.26 and 4.15 min are traces of m/z = 147.12, 85.03 and 129.05, respectively, and correspond to [lysine+H]$^+$, [EG+Na]$^+$ and [DEG+Na]$^+$, respectively.

3.4. Quantification of monomeric products by HPLC–TOF-MS

The low molecular weight products were analyzed by HPLC–ESI-TOF-MS. The TIC chromatogram of the PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ hydrolysate is shown in Fig. 9. The peaks at 1.59, 2.26 and 4.15 min correspond to lysine, EG and DEG, respectively. The peak at 1.68 min are aggregates of sodium formate clusters. In case of pHEMA (300 or 20 kDa) EG is the major product of hydrolysis as compared to DEG and TEG, present as an impurity in the starting material. TEG elutes at 8.40 min. The peak intensities of EG are much lower than that of DEG and TEG (Table 4), because alcohols are not easily charged either in positive or negative mode while DEG can be charged easily due to the presence of its ether group. The three diols form proton [M+H]$^+$, ammonium [M+NH$_4$]$^+$, sodium [M+Na]$^+$ and potassium [M+K]$^+$ adducts. It was attempted to promote the formation of protonated molecule or the ammonium and sodium adducts by adding 0.1% (v/v) formic acid, 0.02% (v/v) ammonia solution (25%) or 0.03% (w/v) sodium iodide to the mobile phase. The peak intensity increased with sodium iodide addition (c.f. Table 4). Therefore, NaI was selected to make sodium adducts for quantification. Lysine preferably makes

Table 4
Relative peak intensities of EG, DEG and TEG in pHEMA hydrolysate with different combinations of formic acid (FA), ammonia (NH$_3$) and sodium iodide (NaI) in the aqueous mobile phase for HPLC–TOF-MS containing 1% organic modifier.

Mobile phase containing	Selected ion	Intensity (Mcps)		
		EG	DEG	TEG
0.1% FA	[M+H]$^+$	0.06	2.60	0.43
0.1% FA + 0.02% NH$_3$	[M+NH$_4$]$^+$	0.08	1.40	0.40
0.1% FA + 0.03% NaI	[M+Na]$^+$	0.51	4.00	0.56

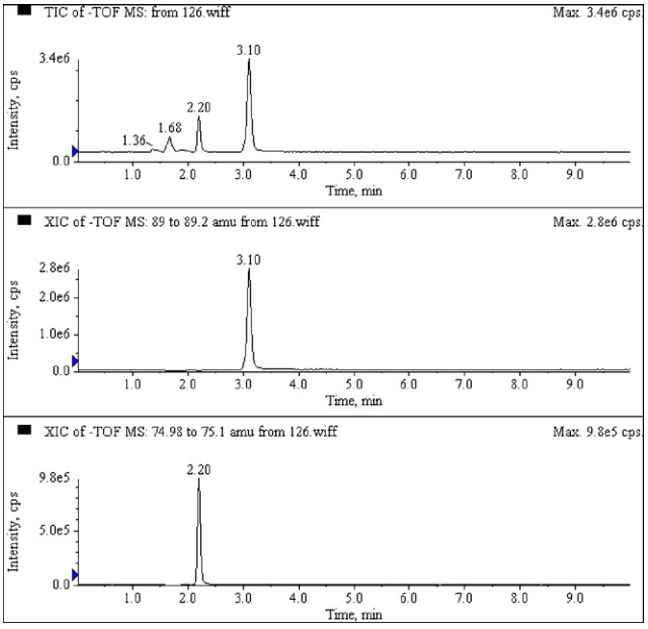

Fig. 10. TIC and XIC chromatogram of PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ hydrolysate. Conditions: negative ESI mode with isocratic elution with water containing 0.1% (v/v) formic acid and 1% (v/v) acetonitrile, flow rate 1.5 mL min^{-1}, column C18 Alltech Prevail (250 mm × 4.6 mm, 5 μm). Peaks at 2.19 and 3.06 min are traces of m/z = 75.01 and 89.02, respectively, and correspond to [GA−H]$^-$ and [LA−H]$^-$, respectively.

[M+H]$^+$ adduct even in the presence of NaI. Lysine remains unretained even at very low concentration of organic modifier. GA and LA in PLGA(50:50)$_{1550}$-diol, PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ were quantified in negative ESI mode as [M−H]$^-$ ion without NaI in the mobile phase and are eluted at 2.19 and 3.06 min (Fig. 10). The standards prepared in water showed peaks of linear dimer (m/z 161.0432) of LA at 7 and 8 min. To avoid this, all the solutions of standards were first prepared in 1 M KOH to convert the dimers into monomers and then diluted up to a dilution factor of 20 with deionized water and pH neutralized with hydrochloric acid.

When quantifying target components in samples one has to take care to avoid matrix overloading. The undetected co-eluting matrix components may reduce the ionization efficiency of the analytes and cause poor reproducibility and accuracy [46,47]. As the lysine elutes at t_0, significant signal suppression occurred and the apparent yield (relative to the assumed structure of the starting materials as in Fig. 1) was 25%. To compensate for this matrix signal suppression, the quantification of components was performed by standard addition giving a yield of lysine of 68%. This indicates that either not all of the etLDI is converted to lysine may be due to the presence of lysine-diacrylate cross-links (Fig. 11B) or some of the cross-link chains are deficient with etLDI. The summary in Table 5 shows that all the hydrolyzed samples were recovered quantitatively with respect to the total amount of sample subjected to hydrolysis, except for pHEMA (20 kDa), which can be explained by incomplete separation (c.f. Fig. 7a) and for PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$, because PEG impurities, ethanol and carbon dioxide, which were produced during the hydrolysis of the last material were not quantified.

In addition to overall recovery, the recovery of the separate hydrolysis products when treating a mixture of the hydrolysis products by the same hydrolysis and analysis procedure, was determined. These recoveries are also indicated in Table 5. Although the recovery of GA is significantly less than 100% the yields are acceptable, considering the small sample size.

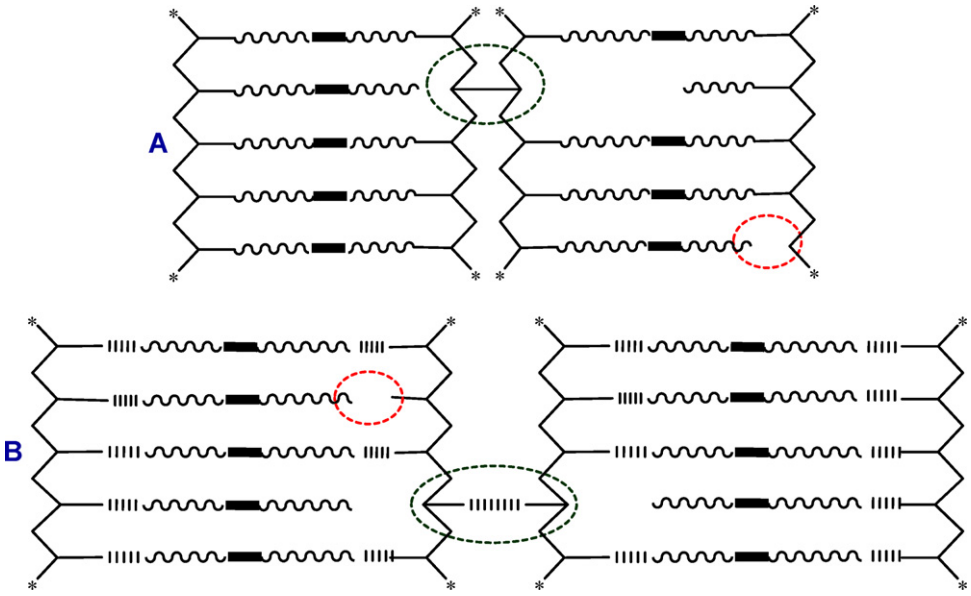

Fig. 11. Proposed chemical structure of (A) PLGA(50:50)$_{1550}$-diol(HEMA)$_2$, (B) PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ based on quantitative hydrolysis results (Tables 1 and 2). Polymethacrylic acid (⁀), ethylene glycol (—), ethyl ester lysine diisocyanate (▮▮▮▮▮▮▮▮▮▮), PLGA (∿∿∿), and diethylene glycol (▬).

Table 5
Summary of the quantitative results of degradation products of biomaterials performed with HPLC–TOF-MS.

Hydrolysate samples	Recovery[b] ± CV (%)	PLGA(50:50)$_{1550}$-diol		pHEMA (300 kDa)		pHEMA (20 kDa)		PLGA(50:50)$_{1550}$-diol(HEMA)$_2$		PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$	
		Amount (mg)	% RSD (n = 2)	Amount (mg)	% RSD (n = 2)	Amount (mg)	% RSD (n = 2)	Amount (mg)	% RSD (n = 2)	Amount (mg)	% RSD (n = 3)
PMA-Na[a]	99 ± 1	–	–	18.97	1	16.81	1	5.26	2	2.43	7
EG	100 ± 3	–	–	6.27	3	7.87	7	2.41	1	1.39	14
DEG	97 ± 2	3.09	4	0.20	5	0.13	2	1.57	6	1.24	4
LA	94 ± 4	27.18	3	–	–	–	–	9.04	4	7.08	4
GA	92 ± 2	22.43	4	–	–	–	–	7.10	4	5.76	2
Lysine	96 ± 4	–	–	–	–	–	–	–	–	1.80	4
Average mass of sample		39.8		20.3		20.2		20.05		20.0	
Average % yield[c]		108	4	98	3	95	5	105	4	92	2

[a] Obtained from SEC-dRI.
[b] Recoveries of each analyte based on control solution.
[c] The average % yield contains the data corrected with mass of the building blocks in the structure.

Table 6 shows the theoretical and the experimental molar ratios among different components of pHEMA, PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$. The theoretical ratios were estimated from Fig. 1 and the experimental ratios are based on the quantitative data presented in Table 5. The results indicate that both pHEMA with 300 kDA MW and PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ are more deficient in EG than pHEMA with 20 kDA MW. This suggests either the presence of cross-linking between two neighboring PMA backbones via esterification and the formation of ethyl diacrylates or the pHEMA as a starting material is partially hydrolyzed. In case of PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ the decrease in the amount of DEG with respect to PMA and EG may either be attributed to the missing cross-links or to the presence of dangling chains without DEG. For PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ the molar ratios between different building blocks are close to those of the ideal structure except the lower amount of lysine compared to DEG.

Based on these experimental ratios between different components, the following structures could be suggested for PLGA(50:50)$_{1550}$-diol(HEMA)$_2$ and PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$ (Fig. 11).

However, it should be stipulated that this aposteriori analysis of the monomers after hydrolysis only determines averages and cannot discriminate between different distributions, e.g. of lysines in side chains, which could have implications for degradation of the material. This stresses that it is imperative to involve analyses in each step of manufacturing, assessing the starting materials and intermediates as well as the final product. The presented method

Table 6
The theoretical and experimental ratio between different components of biomaterials.

Ratio between (Theoretical ratio)	PMA: EG (1:1)	EG: DEG (2:1)	PMA: DEG (2:1)	Lys: DEG (2:1)
PLGA(50:50)$_{1550}$-diol(HEMA)$_2$	1.0: 0.74	2.0:0.77	2.0:0.61	–
PLGA(50:50)$_{1550}$-diol(etLDI-HEMA)$_2$	1.0: 1.0	2.0:1.05	2.0:1.05	2.0: 1.85
pHEMA (300 kDa)	1.0: 0.56	–	–	–
pHEMA (20 kDa)	1.0: 0.83	–	–	–

was not set up to determine the structure of the biomaterial network, but as a balancing check accounting for all the resulting degradation products, since response factors of oligomers are not precisely known, unlike those of the composing monomers.

In degradation studies this method allows quantitative analysis of oligomeric and other intermediates that constitute the majority of degradation products under physiologic conditions, in the second stage of a two step procedure: the first step uses physiologically relevant conditions, while in the second step a fast and complete degradation is executed for quantification of the final products.

4. Conclusions

The current method leads to complete hydrolysis of pHEMA (both high and low MW), PLGA(50:50)$_{1550}$-diol, PLGA(50:50$_{)1550}$-diol(HEMA)$_2$ and PLGA(50:50))$_{1550}$-diol(etLDI-HEMA)$_2$ in 24 h at 120 °C. The Teflon lined microwave vial was helpful to avoid contact of reaction medium with the glass vial. This lead to the complete degradation of biomaterial without the formation of insoluble residues under harsh conditions (high pH, temperature and pressure). NMR proved to be a good analytical technique to monitor the cleavage of bonds in these biomaterials. HPLC–TOF-MS can be utilized to quantify the monomers in the hydrolysis mixture. The origin of bimodality in the MMD of PMA-Na can be inferred from the non-Gaussian distribution of the starting material. This study will be helpful to investigate the hydrolytic degradation and for the compositional analysis of novel polymeric networks including pHEMA as an intermediate product.

Acknowledgements

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