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Enzymatic degradation of the hydrogels based on synthetic poly(α-amino acid)s

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Abstract Biodegradable hydrogels are studied as potential scaffolds for soft tissue regeneration. In this work biodegradable hydrogels were prepared from synthetic poly (α-amino acid)s, poly(AA)s. The covalently crosslinked gels were formed by radical copolymerization of methacryloylated poly(AA)s, e.g. poly[N^5-(2-hydroxy-ethyl)-L-glutamine-*ran*-L-alanine-*ran*-N^6-methacryloyl-L-lysine], as a multifunctional macro-monomer with a low-molecular-weight methacrylic monofunctional monomer, e.g. 2-hydroxyethyl methacrylate (HEMA). Methacryloylated copolypeptides were synthesized by polymerization of *N*-carboxyanhydrides of respective amino acids and subsequent side-chain modification. Due to their polypeptide backbone, synthetic poly(AA)s are cleavable in biological environment by enzyme-catalyzed hydrolysis. The feasibility of enzymatic degradation of poly(AA)s alone and the hydrogels made from them was studied using elastase, a matrix proteinase involved in tissue healing processes, as a model enzyme. Specificity of elastase for cleavage of polypeptide chains behind the L-alanine residues was reflected in faster degradation of L-alanine-containing copolymers as well as of hydrogels composed of them.

1 Introduction

Materials in a form of biodegradable hydrogels are frequently studied as scaffolds for tissue regeneration. The biodegradability is one of important requirements for biomaterials applicable in this field [1] and this feature is often achieved by choosing natural biopolymers, e.g. collagen, for their production. However, in addition to chemical treatment, which is usually needed to improve either mechanical properties or to adjust the rate of degradability, the use of biopolymers isolated from animal or human tissues typically requires a thorough purification to remove components that may, potentially, elicit an immune response or transmit diseases. Therefore, development of fully synthetic biomaterials, structures of which can be tailored on a molecular basis and which could combine advantages of natural and synthetic materials, is highly desirable.

Synthetic poly(α-amino acid)s, poly(AA)s, have been investigated for various pharmacological and medical applications. They can be prepared with a wide range of properties, through which they can mimic some properties of naturally occurring macromolecules and provide biocompatible materials which are degradable with enzymes occurring in both the intracellular and extracellular compartments. As the proteolytic enzymes are specific to the amino acid sequence in the polypeptide chain, the specificity and rate of degradation of poly(AA) hydrogels could be controlled through controlling the poly(AA) composition, e.g. by copolymerization.

High-molecular-weight poly(AA)s can be efficiently prepared by a ring-opening polymerization of cyclic monomers, such as *N*-carboxyanhydrides (NCA) of α-L-amino acids [2, 3]. Poly(γ-benzyl glutamate), PBLG, and related copolymers belong to the most thoroughly studied poly(AA)s. The starting monomer, i.e. γ-benzyl glutamate–NCA, can be prepared in good yield and the resulting polymers have usually convenient solution properties. The water-soluble polymers can be readily prepared from

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PBLG derivatives by conversion of benzyl glutamate units to N^5-(hydroxyalkyl)-L-glutamines, through their aminolysis with hydroxyalkyl amines. In this way, poly[N^5-(2-hydroxy-ethyl)-L-glutamine], PHEG, and copolymers of N^5-(2-hydroxy-ethyl)-L-glutamine, HEG, have been prepared [4, 5]. The hydroxyl groups of PHEG can be used for additional side-chain modification, e.g. methacryloylation [6].

The degradability of PHEG derivatives, i.e. HEG homopolymers and copolymers, by various enzymes was studied previously. The size and composition of degradation fragments after incubation of PHEG copolymers containing L-leucine and L-phenylalanine with lysosomal enzymes were analyzed by Chiu et al. [7] using electrospray mass spectroscopy. A range of di- to pentapeptides was found as the final degradation products after incubation with model enzymes, such as papain and chymotrypsin, with tri- and tetrapeptides being the major components. The degradation of the same polymers by pronase produced individual amino acids as the major degradation products [8]. These results are not surprising, because pronase actually contains a variety of enzymes, both endo- and exopeptidases. Thus, it was repeatedly confirmed that, in principle, poly(AA)s such as PHEG and related copolymers can be degraded in the body to low-molecular-weight fragments, eventually, to monomer units.

The objective of this study was to synthesize series of statistical copolymers of HEG with various content of two other monomers, such as methacryloylated L-Lys (MA-L-lysine) and L-Ala, and to investigate the effect of copolymer composition on their biodegradability and biodegradability of hydrogels prepared from them. Hydrogels were prepared by radical crosslinking copolymerization of methacryloylated poly(AA)s as multifunctional crosslinker and HEMA as an additional methacrylic monomer. This technique allows for preparation of well-defined covalently crosslinked hydrogels, in which the crosslinking density, permeability and rheological properties can be well controlled [6]. The hydrogels of this type could be well suited as biomaterials for tissue engineering scaffolds [9]. As the scaffolds are temporary substitutes for extracellular matrix (ECM) structures, the susceptibility of the scaffold to be degraded by processes regularly occurring in regenerating tissue would be of particular advantage. In this study we use elastase as a model enzyme. Elastase is a matrix proteinase (MMP-12), which is involved in tissue healing processes and remodeling of ECM. Because elastase cleaves preferentially the peptide bonds next to the L-alanine residues [10, 11], we investigated the effect of L-alanine units in the copolymers on the promotion of polymer degradation by this enzyme.

2 Experimental

2.1 Materials

Tetrahydrofuran, *N*,*N*-dimethylformamide, dimethyl sulfoxide, chloroform, hexane (all from Lach-Ner, Czech Rep.) and 2-aminoethanol (from Fluka) were dried with appropriate drying agents, distilled and stored over a molecular sieve. Disodium hydrogen phosphate dodecahydrate, potassium dihydrogen phosphate (both from Lach-Ner), Tris (hydroxyethyl)aminomethan, diisopropyl amine (DIPA), sodium carbonate, sodium hydrogen carbonate (all from Fluka) were of an analytical grade and were used as obtained. TNBS (2,4,6-trinitro-benzene sulphonic acid, sodium salt), L-α-alanine, triphosgene, SLAPNA (Suc-(L-Ala)$_3$-*p*-nitroanilid), β-alanine and elastase (from porcine pancreas, type III) were purchased from Sigma. Poly(ethylene glycol) standards were purchased from Polymer Standard Service (Germany). 2-hydroxyethyl methacrylate (HEMA) (from Röhm GmbH, Germany) was used without purification. 2,2′-azobisisobutyronitrile (AIBN, from Fluka) was recrystallized from ethanol prior use. γ-Benzyl L-glutamate [12], ε-phthaloyl-L-lysine [13], *N*-acetylglycine [14] and *N*-methacryloyloxy succinimide were synthesized according to the procedures from literature with minor modifications [15]. Q-water (Milli-Q® + Q Gard® 1, Millipore Corporation, USA) was used for all operations with elastase.

2.2 Synthesis of monomers

NCAs of L-alanine, ε-phthaloyl L-lysine, γ-benzyl L-glutamate and *N*-acetylglycine were prepared by reaction respective amino acid derivatives with triphosgene [16]. Briefly, the amino acid was suspended in anhydrous tetrahydrofuran (or dioxane) and kept at 45–55°C under inert gas (argon or nitrogen) and triphosgene (10–20% mole excess) was added. The reaction was carried out for 1–2 h with stirring. The reaction mixture was filtered and tetrahydrofuran was removed partially by rotary evaporation. Crude NCA was crystallized with addition of chloroform and hexane. Recrystallization was carried out from ethyl acetate and hexane. Purity of each monomer was characterized by melting point, elementary analysis (Perkin-Elmer 2400, USA) and IR spectroscopy (Perkin-Elmer Paragon 1000PC, USA).

2.3 Synthesis of macromonomers

Macromonomers for gel formation, i.e. methacryloylated poly(AA)s, were synthesized according to Fig. 1. First, the copolymers of γ-benzyl-L-glutamate, ε-phthaloyl-L-lysine and L-alanine were prepared by polymerization of calculated amount of respective NCAs in *N*,*N*-dimethylformamide

(0.2 mol/l), initiated with *N*-acetylglycine-NCA as an initiator and DIPA as a catalyst [17]. The polymerizations took 4 h at laboratory temperature (22°C). In all cases, a small sample of the reaction mixture was withdrawn, precipitated in methanol and the polymerization yield of product (Fig. 1I) was calculated. In the rest of polymer, the glutamate esters were aminolyzed without polymer isolation by adding ten times mole excess of 2-aminoethanol into the reaction mixture and heating it to 50°C for 3 days. The solution of the resulting aminolyzed product, a terpolymer of HEG, L-lysine and L-alanine (Fig. 1II), was neutralized with acetic acid, dialyzed against water (Spectra/Por®, dialysis tubing), concentrated by ultrafiltration (Amicon® 8400 with YM3 filter with a cut-off 3.000 Da, Millipore Corporation, USA) and lyophilized (Lyovac GT 2, Leybold AG, Germany).

The terpolymer of HEG, L-lysine and L-alanine was dissolved in dry *N*,*N*-dimethylformamide (5 wt%) and reacted with *N*-methacryloyloxy succinimide (50% mole excess with respect to the content of L-lysine unit in the copolymer). The reaction was carried out for 2 days at room temperature. The resulting methacryloylated water-soluble polymer (Fig. 1III) was then purified by dialysis, ultrafiltration and lyophilized.

2.4 Characterization of polymers

All copolymers were analyzed by ^{1}H NMR spectroscopy (Bruker Avance DPX 300 MHz) in DMSO for water-insoluble copolymers, and in D_2O with DSS as an internal standard for water-soluble copolymers. The content of MA-L-lysine in macromonomers was calculated from the ratios of the integrated peaks of methacryloyl methylene to α-methine protons of the polypeptide backbone (see Fig. 2).

The NMR analysis of copolymer composition was complemented by amino acid analysis, AAA (Acc.Tag Chemistry Package from Waters, USA; Nova-Pak C_{18} column, according to ref. [18]). In addition, the presence of free amino groups of L-lysine units was confirmed by the reaction with TNBS [19]. The molecular weight averages of water-soluble copolymers were determined by SEC analysis (TSKgel G3000 PWXL column and guard column TSKgel PWXL Guardcol from Tosoh Bioscience, Japan and differential refractometer from Millipore® R401 from Waters Associated, USA). PEG standards were used for calibration and 0.15 M phosphate buffer (pH 7.80) as a mobile phase. The flow rate was 0.8 ml/min. The calibration was recalculated to PHEG using universal calibration concept [20] with the Mark–Houwink coefficients $K_{PEG} = 0.050$ cm^3/g, $a_{PEG} = 0.67$ for the PEG standards [21] and $K_{PHEG} = 0.015$ cm^3/g, $a_{PHEG} = 0.7$ for PHEG [22].

2.5 Preparation of hydrogels

Water-soluble macromonomers (MC-0, MC-6 and MC-12) with multiple methacryloyl groups per average polymer chain were used as a crosslinker in radical copolymerization with HEMA using AIBN as an initiator (see Fig. 3). Each reaction mixture contained 17 wt% of macromonomer and 3 wt% of HEMA in water and the concentration of AIBN in the mixture was 0.0336 wt%. Briefly, the separate stock solutions of macromonomers and HEMA were deoxygenated by bubbling with nitrogen and evacuation. AIBN was dissolved in HEMA and the required amount of

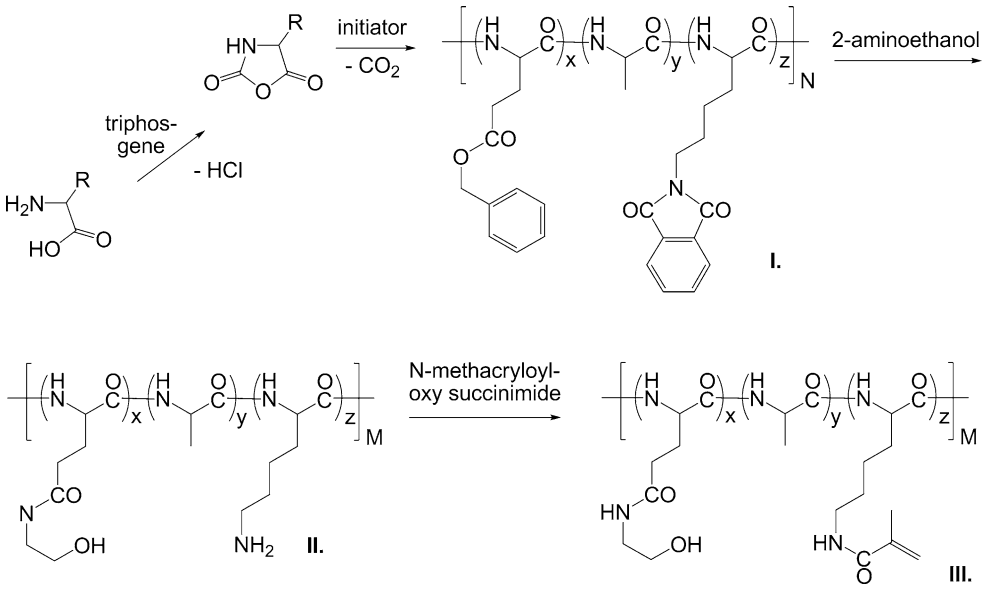

Fig. 1 Synthesis of macromonomers

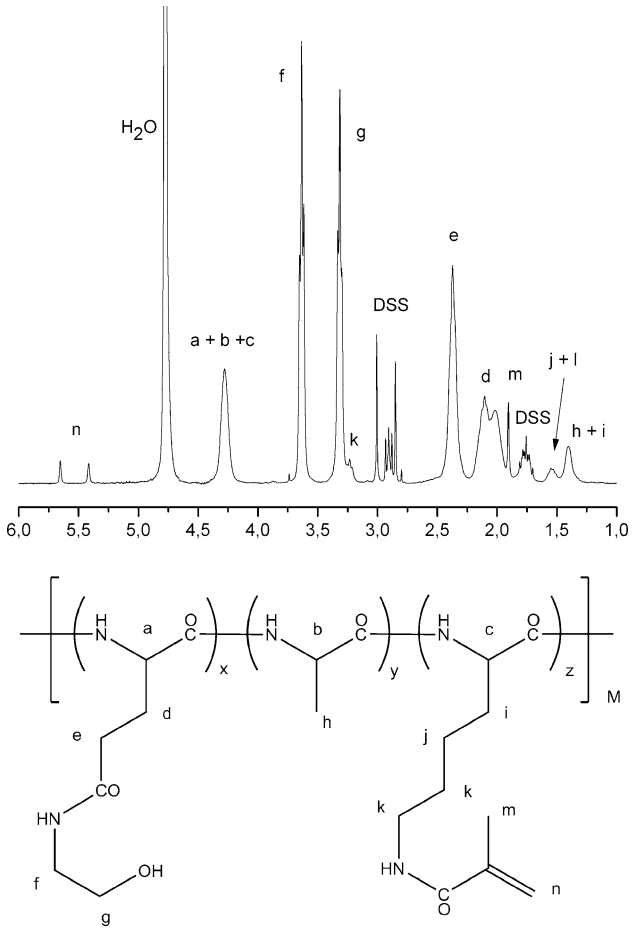

Fig. 2 Example of ^{1}H NMR spectrum of methacryloylated copolymer (MC-12) for calculation of content of MA-L-lysine units. The *letters* mark the peaks of respective protons, shown in the molecular formula under the spectrum

this solution was added into the degassed stock solution of the macromonomer. The polymerization mixture was injected into glass vials under nitrogen, the vials were sealed with a rubber stopper and kept at 62°C. After 17 h the vials were broken and gels were extensively washed with water and swollen to equilibrium in water for 10 days.

2.6 Enzymatic degradation of polymers and gels

The degradation by elastase for polymers with different composition was compared using the same enzymatic activity of elastase in each series of polymers to be compared. The elastase activity was determined spectrophotometrically using SLAPNA as a testing substrate according to the method described in the literature [23]. The activity of enzyme preparation was 3.5 ± 0.1 U/mg under standard conditions (0.1 M Tris–HCl, pH 8.00, 25°C) and 3.1 ± 0.1 U/mg in Sörensen buffer (0.15 M phosphate buffer, pH 7.80, 25°C).

The degradation progress of linear water-soluble poly (AA)s by elastase was evaluated from the change of the polymer number average molecular weight, determined from SEC analysis [8], after the treatment with enzyme under the conditions as above. Briefly, copolymers were dissolved in Sörensen buffer, the sample of the solution was kept at 37°C and a measured amount of the stock solution of enzyme in the same buffer, pre-equilibrated to the same temperature, was added. The concentration of presumed enzyme-cleavable peptide sequences, i.e. L-alanine units, was kept constant (0.226 mg/ml of L-alanine, about 3.3–5.0 mg/ml of copolymers, concentration of L-alanine free copolymer was 0.33 mg/ml) in all series of copolymers under testing and the concentration of enzyme was 0.1 mg/ml (0.35 U/ml) in all series. The incubation with enzyme was carried out for 24 h, during which several samples were withdrawn for SEC analysis. The degradation in the withdrawn samples was stopped by heating the sample to the boiling point for 5 min. Denatured enzyme was filtered off (0.45 μm nylon filter, Acrodisc®) and the sample was frozen-stored until the SEC analysis (no difference between the stored and immediately measured samples was observed).

The degradation of hydrogels was followed by the change of relative swelling of the gel sample during its incubation with elastase. The starting weights of cylindrical samples were about 0.75 g. Concentration of enzyme was 0.2 mg/ml and a fresh enzyme was added each 24 h in the same concentration to compensate for its autodigestion.

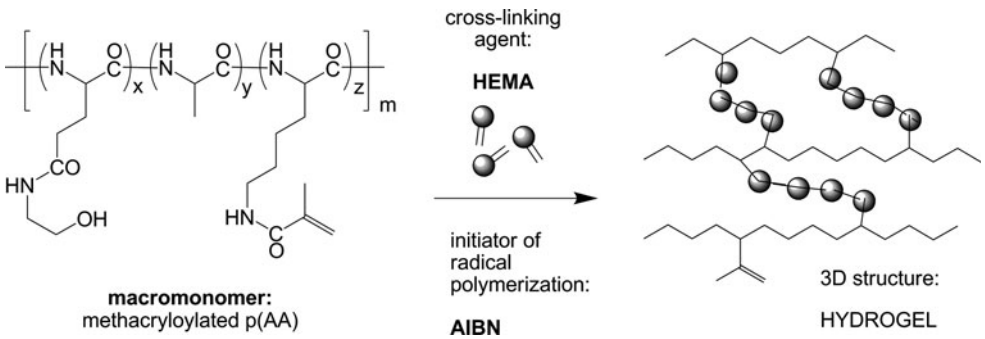

Fig. 3 Preparation of hydrogels from methacryloylated PHEG

Activity of enzyme in incubation medium before addition of fresh enzyme was 3.1–3.8 U/mg.

3 Results

The copolymers were prepared by ring-opening polymerization of *N*-carboxyanhydrides of protected derivatives of respective amino acids. The side-chains protecting groups, i.e. benzyl of γ-benzyl-L-glutamate and phthaloyl group of ε-phthaloyl L-lysine were removed in one step by aminolysis with 2-aminoethanol, thus yielding linear water-soluble polymers. The methacryloyl groups in the copolymer side chains were introduced by the reaction of *N*-methacryloyloxy succinimide with free ε-amino groups of L-lysine units in copolymers. The characteristics of prepared copolymers are summarized in Tables 1 and 2. The composition of all copolymers was determined by amino acid analysis and, besides, the content of MA-L-lysine units in methacryloylated copolymers was determined by ^{1}H NMR. The number in the sample code indicates a theoretical mole percent of L-alanine units in the copolymer, based on the composition of the polymerization mixture. The projected content of L-lysine units was 12 mol percent in all copolymers. It is evident that composition of synthesized copolymers follows the monomer ratio in the polymerization feed. The content of MA-L-lysine in copolymers after methacryloylation corresponds rather to content of free L-lysine amino groups before methacryloylation, as measured by TNBS method. The molecular weight averages of copolymers upon methacryloylation determined by SEC (for MC-12 only) appear to be reduced, what might be explained rather by lower polymer coil expansion of the methacryloylated copolymer than by degradation of the polymer chain during methacryloylation.

L-lysine was incorporated into the polymer backbone, to secure methacryloylation through amide bond only, because of greater stability of the methacryl amide bond compared to the ester bond that would result from derivatization of PHEG with methacryloyl chloride. The stability of this bond is advantageous with respect to the further hydrogel modification, e.g. for fabrication of macroporous gel scaffolds using PLLA-fiber-leaching method [9]. The presence of nucleophilic α-amino group of L-lysine makes it possible to carry out their selective methacryloylation through aminolysis of methacrylic acid *N*-hydroxysuccinimide esters.

The time course of degradation of linear poly(AA)s by elastase for copolymers with different content of L-alanine units is shown on Fig. 4. Typical error of these measurements was smaller than 5%. The effect of L-alanine content on the rate of poly(AA) degradation is evident. The degradation rate increased with the increasing content of L-alanine in the copolymer chain. The relative molecular weight averages of all samples decreased in a monotonous

Table 1 The molecular weights and the mole fractions of constitutional units (in %) of copolymers as obtained from indicated analytical methods

Sample code[a]	Yield [%][b]	L-alanine	L-lysine		Mw	Pd
		AAA	TNBS[c]	AAA	SEC	
AC-0	88	0.5	5.5	11.2	17 100	1.63
AC-6	93	9.6	4.9	11.0	17 600	1.53
AC-12	69	15.0	5.9	10.8	19 100	1.51

[a] *AC* polypeptides before methacryloylation, numbers give the theoretical mole fraction of L-alanine units (in %) in the copolymer (amount of L-alanine in the feed)

[b] A total yield after polymerization and aminolysis

[c] Determination of free amino groups by TNBS method

Table 2 Mole fractions of methacryloylated L-lysine units (MA-L-lysine, in %) and molecular weights of methacryloylated copolymers

Sample code[a]	Yield [%][b]	MA-L-lysine		Mw/pd
		NMR	AAA	SEC
MC-0	27	5.3	–	ND
MC-6	23	4.8	–	ND
MC-12	27	6.6	–	13 500/1.66

[a] *MC* methacryloylated polypeptides, numbers give the theoretical mole fraction of L-alanine units (in %) in the copolymer (amount of L-alanine in the feed)

[b] Total yield from polymerization to methacryloylation

ND not determined

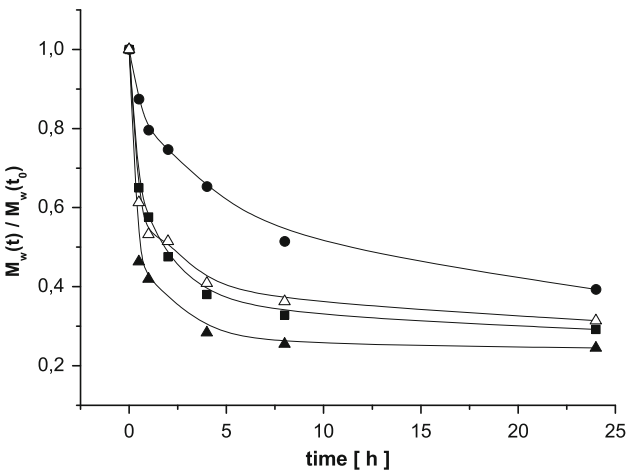

Fig. 4 Relative change of molecular weight of polypeptides during incubation with elastase (from SEC); *filled circles* (AC-0), *squares* (AC-6) and *triangles* (AC-12), *unfilled triangles* (MC-12); *error* < 5%

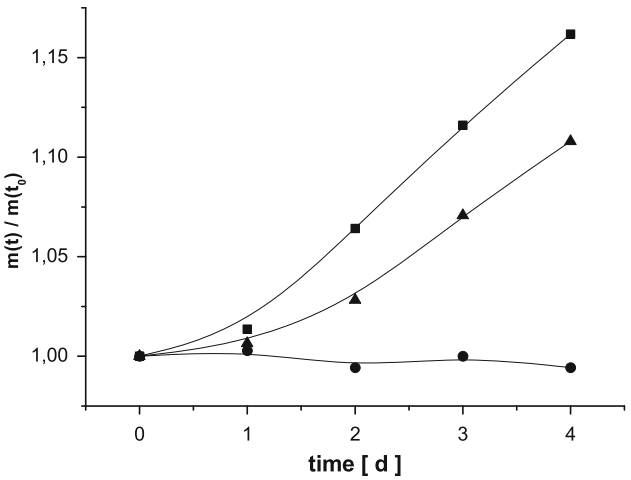

Fig. 5 The relative swelling of hydrogels made from copolymers with different content of L-alanine units when incubated with elastase; *circles*, *triangles* and *squares* represent hydrogels HG-0, HG-6 and HG-12 made from methacryloylated copolymers MC-0, MC-6 and MC-12, respectively; *error* < 2%

way and the degradation resulted in products with a limited molecular weight, tending to the estimated size of average polymer segments between L-alanine units.

The effect of copolymer composition on enzymatic degradation of the hydrogels prepared from copolymers described above is shown on Fig. 5. The degradation of poly(AA) chains should result in the decrease of the gel crosslinking density, which, in turn, should be reflected in the increased gel swelling. In Fig. 5, the progress of degradation of gels with different composition was evaluated as increased swelling of the gel, which was followed by repeated weighing. Though the error of the method itself is very low (typically smaller than 2%), we are aware that the swelling method provides only an approximate estimate of the degradation rate. Nevertheless, we believe the data clearly show the difference in the gel response depending on the presence of L-alanine units.

Both hydrogels (HG-6 and HG-12) made of copolymers with L-alanine, were degraded by elastase as revealed from the increase of their swelling in presence of the enzyme, although the effect of L-alanine content on the degradation rate cannot be exactly quantified. At initial stage, the hydrogel with lower L-alanine content (HG-6) was degraded even faster than HG-12, however, after about 2 days, the slopes of swelling curves of both hydrogels became approximately equal. The possible explanation of these peculiarities is discussed below.

4 Discussion

The presented results demonstrate the feasibility of using polyfunctional macromonomers based on methacryloylated synthetic poly(α-amino acid)s as precursors for preparation of crosslinked hydrogels. We have also demonstrated that the enzymatic degradation for both, macromonomers and resulting hydrogels, can be affected by the composition of starting copolypeptides. In the presented system the susceptibility of PHEG to specific degradation by elastase was rendered by incorporation of L-alanine units as a minor component of the copolymer with 2-hydroxyethyl glutamine (HEG).

These findings correspond well with previous studies on enzymatic degradation of synthetic poly(α-amino acid)s. The effect of enzymes with various specificity on PHEG, and HEG copolymers with L-alanine, L-valine, L-leucine, L-phenylalanine and other amino acids and their derivatives, was investigated by several researchers [6–8, 24–26]. Plant thiol proteases (papain, ficin and bromelain) were usually selected as the model enzymes because of their specificities similar to cathepsin B, a lysosomal thiol endopeptidase. The latter enzyme participate significantly in a wound healing and have a broad specificity [27–29] with preferences both for basic amino acids, such as L-lysine and L-arginine or L-glutamine, and large hydrophobic amino acids, such as L-phenylalanine, L-alanine, L-leucine or L-valine, at P_2 and P_1' residue sites of the substrate. No effect of trypsin on degradation of PHEG was observed [24].

The effect of structure and content of hydrophobic side-chains on degradability of PHEG derivatives by chymotrypsine A was examined in our laboratory previously [24]. The copolymer of 2-hydroxyethyl glutamine (HEG) and L-phenylalanine (L-Phe) was the most degraded among HEG copolymers because of specificity of chymotrypsin A for the peptide bond next to the phenylalanyl residues. The degradation rate was proportional to the content of L-Phe in the polymer chain. Few authors demonstrated the feasibility of degradation of cross-linked gels based on poly(AA)s, specifically PHEG and its copolymers [5, 27, 30–33]. The degradation of PHEG derivatives by elastase was not yet described.

In our system, L-alanine was chosen as the enzyme specificity-modifying structure, assuming that it would render degradability of the copolymer by elastase, in accord with the known elastase specificity [10, 11]. Indeed, the effect of L-alanine content on the degradation rate was clearly shown. A minor degradation of copolymer without L-alanine (AC-0) was also observed. This fact can be attributed to presence of impurities in the elastase preparation that might have a broader enzymatic specificity. A typical impurity of elastase, such as trypsin, cleaves peptide bonds behind basic amino acids, such as L-lysine and L-arginine [34]. Therefore, presence of L-lysine structural units in the copolymer can be responsible for contribution of this type of enzymatic activity. In this respect, it is worth noticing that the methacryloylated copolymer (MC-12), in

which the original basic L-lysine units were blocked by methacryloylation, was degraded in significantly lower rate compared with unmodified copolymer with the same content of L-alanine (AC-12). In addition, the hydrogel formed from the macromonomer without L-alanine (copolymer MC-0), HG-0, does not show any degradation by elastase, despite that the linear copolymer (AC-0) was slightly degraded. This finding supports the explanation given above for linear copolymer (AC-0) because, in hydrogels, L-lysine units are incorporated into cross-links, hence could not contribute to the degradation.

One should be aware that evaluation of the progress of degradation by following the increase of swelling degree provides only a qualitative measure of degradation. It is known that the onset of degradation of hydrogel in the bulk and, subsequently, its effect on the gel swelling depends primarily on the ability of enzyme to penetrate into the gel network, which, in turn, is controlled by crosslinking density of the gel [35]. Although the macromonomers were synthesized to have the same content of MA-L-lysine units, hence expecting a similar crosslinking density of both samples, in reality, the degree of methacryloylation of colymer MC-12 was higher compared to MC-6 as shown in Table 2, which could contribute to higher crosslinking density of HG-12 compared to HG-6. In addition, the efficiency of crosslinking reaction of the copolymer MC-12 with higher content of L-alanine, i.e. with higher fraction hydrophobic side chains, could be also higher than that of MC-6 because of higher interchain interactions. In that case, the onset of bulk degradation and hence the increase of swelling will be delayed because of longer time needed for the enzyme to penetrate in the bulk of the gel network. A more detailed study, relating the degradation rate to directly measured crosslinking density of the gel, is underway.

Our future interest is to use these biodegradable polymers for preparation of highly porous 3D-hydrogel structures—scaffolds, that can be used, e.g. for regeneration of cartilage or for construction of nerve guides. In addition to adjustable enzymatic degradability, we can also benefit from good modification options that synthetic polyAAs offer, to introduce covalently bound specific peptide motives, e.g. RGDS, through which interactions of cells via their integrin receptors with synthetic polyAA hydrogels can be promoted [36].

5 Conclusions

Covalently crosslinked hydrophilic gels based on synthetic poly(AA)s which are selectively degradable by tissue metalloproteinase enzyme, e.g. elastase, have been developed. The susceptibility of the gel towards the elastase degradation has been incurred through introduction of L-alanine units using statistical copolymerization.

Strong dependence of the degradation rate of poly(AA)s on the content of L-alanine in the polymer chain has been found. The degradation rate increased with the increasing content of L-alanine in the polymer chain, corresponding to known elastase specificity. A minor degradation of the copolymer without L-alanine was attributed to presence of impurities with broader specificity in the elastase sample.

Enzymatically degradable hydrogels based on synthetic poly(AA)s, the degradation of which by specific tissue enzymes can be controlled by composition of poly(AA) chains, thus show one of the key characteristics required for tissue-engineering biomaterials.

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